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(54) Title: INHIBITION OF CELL ADHESION BY CHEMICALLY-DEFINED OLIGOSACCHARIDES, THEIR DERIVATIVES, MIMETICS, AND ANTIBODIES DIRECTED THERETO

(57) Abstract

Many tumor-associated and leukocyte-associated carbohydrate antigens function as adhesion molecules, recognized by lectins (carbohydrate-protein interaction) or complementary carbohydrates (carbohydrate-carbohydrate interaction). Common structures are found in the tumor-associated and leukocyte-associated antigens. Metastatic potential of tumor cells as well as transendothelial migration of leukocytes was suppressed by agents, or combinations of agents of the groups: (a) carbohydrate antigens; (b) antibodies directed to those antigens; (c) oligosaccharide components of those antigens; (d) conjugates of the antigens or oligosaccharides; and (e) mimetics of the antigens or oligosaccharides. Disclosed are oligosaccharides and derivatives thereof which inhibit cell adhesion and aggregation mediated by P-selectin (GMP-140) and E-selectin (ELAM-1). Effective agents for those purposes include hybrid sugars comprising multiple epitopes, such as Le^x/SLe^x, combination of individual sugars that comprise a hybrid sugar, which may be presented on liposomes, and antibodies or combinations of antibodies directed thereto.

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Inhibition of Cell Adhesion by Chemically-Defined Oligosaccharides, Their Derivatives, Mimetics, and Antibodies Directed Thereto

Cross-Reference To Related Applications

This application is a continuation-in-part of pending U.S. Application Ser. No. 07/950720 filed 25 September 1992; which is a continuation-in-part of pending U.S. Application Ser. No. 07/836978 filed 19 February 1992; which is a continuation-in-part of pending U.S. Application Ser. No. 07/789969 filed 12 November 1991; which is a continuation-in-part of pending U.S. Application Ser. No. 07/724983 filed 2 July 1991; which is a continuation-in-part of U.S. application Serial No. 07/575539 filed 30 August 1990 (abandoned).

All five applications expressly are incorporated herein by reference.

Technical Field

The present invention is directed generally to the inhibition of tumor cell metastases and invasiveness and of inflammatory processes based on the inhibition of adhesion of tumor cells or inflammatory leukocytes to specific types of cells. More specifically, the invention is directed to such inhibition through the use of tumor-associated carbohydrate

antigens, leukocyte-associated carbohydrate antigens, oligosaccharide derivatives thereof, mimetics f the tumor-associated carbohydrate antigens, leukocyte-ass ciated carbohydrate antigens and antib dies directed to the tumor-associated carbohydrate antigens.

Background of the Invention

Despite enormous investment of financial and human resources, cancer remains one of the major causes of death. Current cancer therapies cure only about half of all patients who develop a malignant tumor. In most human malignancies, metastasis is the major cause of death.

Metastasis is the formation of secondary tumor colonies at one or more distant sites. Metastasis is a multistep process of which tumor invasion is the first step. Tumor cells locally invade host tissue barriers, such as the epithelial basement membrane, to reach the interstitial stroma where the tumor cells gain access to blood vessels (or lymphatic channels) for further dissemination. After invading the endothelial layer of the vessel wall, the circulating tumor cells are dislodged into the circulation and arrest in the precapillary venules of the target organ by adhering to endothelial cell lumenal surfaces or exposed basement membranes. The tumor cells again invade the vascular wall to enter the organ parenchyma. Finally, the extravasated tumor cell grows in a tissue different from where the tumor originated.

In most human malignancies, distant metastases often are too small to b det cted at the time th primary tumor is treated. Furthermore, widespread initiation of metastatic colonies usually occurs before clinical symptoms f m tastatic disease are evident. The size of the metastases, age of the patient, dispersed anatomic location and heterogeneous composition all are factors that hinder surgical removal of tumors and limit th concentration of anticancer drugs that can be delivered to the metastatic colonies.

Due to difficulties in the current approaches for treating and preventing metastases, there is a need in the art for improved methods and compositions capable of inhibiting the metastasis potential of tumor cells. The present invention fills those needs and further provides other related advantages.

On the other hand, there are common mechanisms between the initiation of the inflammatory process and metastasis. For example, both processes are triggered by adhesion of cells, leukocytes in the former case and tumor cells in the latter, to microvascular endothelial cells followed by transendothelial migration of the leukocytes or tumor cells into the tissu spaces. Both processes are enhanced by activating platelets. Both processes are mediated strongly by specific types of carbohydrates, such as tumor associated carbohydrate antigens (TACA) or leukocyte associated carbohydrate antigens (LACA). Some TACA's share structures with LACA's.

The instant invention is directed to and based on the inhibition of cell adhesion, for example, through TACA's or LACA's, using, for exampl , antibody th reto.

Summary of the Invention

Briefly stated, the instant invention provides compositions and methods of inhibiting metastatic potential and invasiveness of tumor cells based on blocking tumor cell adhesion by carbohydrate structures or antibodies directed thereto. The instant invention also relates to compositions and methods f inhibiting inflammation potential of leukocytes based on blocking leukocyte adhesion by carbohydrate structures or antibodies directed thereto. The rationale for the approach is to block (a) carbohydrate to carbohydrate interaction; (b) carbohydrate to selectin interaction; or (c) both. For example:

i) In model experiments with mouse melanoma B16 variants with high and low metastatic potential, high metastatic variants, BL6 and F10, express more GM3 than low-metastatic or non-metastatic variants, F1 or Wa4. Adhesion of high metastatic variants to endothelial cells is greater than with low metastatic variants and the adhesion is inhibited by Me-β-lactoside, GM3 or LacCer (each within liposomes) or other lactoside derivations. The sugars and derivatives also inhibit B16 melanoma metastatic potential. Such is an example of (a) above, that is, interfering with a carbohydrate to carbohydrate interaction.

ii) As to human cancer, patients whose primary tumors express defined tumor-associat d carbohydrate antigens, such as $H/Le^y/Le^b$ (defined by mon clonal antibody MIA-15-5), sialosyl Tn (defined by monoclonal antibody TKH2) or sialosyl-Le^x (defined by monoclonal antibody FH6, SNH3 or SNH4), had a much shorter survival rate than those patients whose primary tumors do not express or which weakly express those antigens.

iii) Those tumor-associated carbohydrate antigens (GM3 in the mouse melanoma model and $H/Le^b/Le^y$, sialosyl-Le^x or sialosyl-Tn in human tumors) are essentially adhesion molecules which are recognized by targ t cells, particularly platelets or endothelial cells. Such is an example of a combination approach, that is, interfering with (a) and (b).

iv) Interaction of tumor cells with endothelial cells and platelets is mediated by LECCAM (or selectin), ELAM-1 or GMP-140, which are expressed on activated endothelial cells and activated platelets. Sialosyl-Le^x antigen has been known to be recognized by those LECCAM's. Such is an example of (b), affecting a carbohydrate to selectin interaction.

v) GMP-140, whose expression on platelet or endothelial cells is induc d by thrombin, ADP or (AMP) phorbol ester, may play an imp rtant r le in

platelet-tumor cell interaction and mediate tumor cell metastases. While the epitope recognized by that selectin was identified previ usly as sialosyl-Le^x (Polley et al., Proc. Natl. Acad. Sci. 88:6224, 1991), it has been found that sialosyl-Le^a (also known as monosialosyl-Le^a I), monosialosyl-Le^a II (a positional isomer of sialosyl-Le^a) and disialosyl-Le^a also are recognized by GMP-140. GMP-140 binds to sialosyl Le^a better than to sialosyl-Le^x. Such is another exampl of process (b).

vi) ELAM-1, whose expression on endothelial cells is induced by interleukin-1, TGF-β, TNF-α or lipopolysaccharide, may play an important role in endothelial cell-leukocyte and endothelial cell-tumor cell interaction, mediate tumor cell metastasis, mediate endothelial cell-leukocyte interactions and mediate transendothelial migration of leukocytes and tumor cells. While the epitopes recognized by that selection previously were identified as sialosyl-Le^x and sialosyl-Le^a (Phillips et al., Science 250:1130, 1990; Berg et al., J. Biol. Chem. 266:14869, 1991; Takada et al., Biochem. Biophys. Res. Commun. 179:713, 1991), it has been found that the selectin epitopes also are internally sialylated, penultimate fucosylated type 1 or type 2 chains, such as mon sialosyl-Le^a II and disialosyl-Le^a, particularly in a dynamic flow system. But the binding phenomenon is

vibrant. Under static or low sh ar stress dynamic conditions, ELAM-1 (also known as E-sel ctin) r cognizes primarily $\alpha 2 \rightarrow 3$ sialylated and $\alpha 1 \rightarrow 3$ or $\alpha 1 \rightarrow 4$ fucosylated carbohydrates, such as SLe^x and SLe^a. However, under middle to high shear stress dynamic conditions, molecules having formulae (I) or (II), see, for example, Figure 20, such as Le^x/SLe^x, play an important role in providing high affinity binding sites to E-selectin. That role is particularly evident under high shear stress conditions.

vii) Human colon tumor cells showing differential expression of metastatic potential in nude mice showed a close correlation with the expression of sialosyl-Le^x, i.e., cells with high metastatic potential expressed high levels of sialosyl-Le^x and vice versa.

viii) Adhesion of E-selectin-expressing cells to SLe^x is enhanced greatly when SLe^x is mixed in liposomes with various quantities of Le^x. Hence, greatly enhanc d adhesion was observed not only with hybrid SLe^x/Le^x, but also with mixed glyco-liposomes with SLe^x and Le^x.

ix) Human endothelial cells are characterized by high expression of H (Fuc$\alpha 1 \rightarrow 2$Gal) and many types of human cancers are characterized by expression of Le^y, H or Le^b d fined by monoclonal antibody MIA-15-5.

Interaction of H with Le^y or H with H has been established clearly, therefore, those human tumors expressing $H/Le^y/Le^b$ may adhere to H-expressing endothelial cells which are mediated by Le^y-H or H-H interaction. Such is an example of process (a), that is affecting a carbohydrate to carbohydrate interaction.

x) Monoclonal antibody MIA-15-5 directed to $H/Le^y/Le^b$ inhibited lung metastasis of highly metastatic F10 and BL6 variant cells in the mouse. Furthermore, monoclonal antibody FH7 directed to disialosyl-Le^a and monosialosyl-Le^a II inhibited adhesion of human cancer cells expressing those antigens in a dynamic flow system.

Based on those and other various observations and considerations, the instant invention provides the following:

a) Compositions and methods for inhibiting tumor cell metastasis based on tumor cell adhesion mediated by carbohydrate antigen by such oligosaccharides comprising GM3, H, Le^y, Le^b, monosialosyl-Le^x (SLe^x), Le^a, Le^x, hybrid sugars, such as, Le^x/SLe^x hybrids (Structure 1 in Figure 20), monosialosyl-Le^a I (SLe^a), monosialosyl-Le^a II, sialosyl Tn, lactosyl and other structures as depicted in structures 1-14, in Example 3.

b) Compounds, such as those set forth in Figure 20, which can be Le^x/SLe^x hybrids, or an appropriate mixture of the relevant components, such as Le^x and SLe^x, provide high affinity adhesion binding sit s, particularly under high shear stress conditions in a dynamic flow system. Hence, such compounds block E-selectin-mediated adhesion of tumor cells or leukocytes to endothelial cells.

c) Oligosaccharide derivatives based on those structures and linked to an appropriate carrier.

d) Oligosaccharide derivatives whose sugar structures are modified appropriately showing better blocking activity of tumor cell adhesion based on oligosaccharide-lectin (selectin; LECCAM) or oligosaccharide-oligosaccharide interaction.

e) Utilization of antibodies recognizing those oligosaccharides comprising and representing tumor-associated carbohydrate antigens also may inhibit tumor cell adhesion to endothelial cells, platelets or target cells, and may inhibit metastasis.

e) Utilization of combinations of antibodi s recognizing those oligosaccharides involved in cell adh sion and representing tumor-ass ciated antigens.

Thus, in one aspect of the instant invention, a method for inhibiting tumor cell metastasis potential or inflammation within a biologic preparation is provided. The method comprises incubating the biologic preparation with at least one agent selected from the group consisting of (a) tumor-associated carbohydrate antigens (or leukocyte-associated carbohydrate antigens) that exhibit differential prognostic significance, (b) antibodies that specifically bind to those antigens, (c) oligosaccharide components of those antigens, (d) conjugates of those antigens or oligosaccharides and (e) mimetics of the tumor-associated carbohydrate antigens (or leukocyte-associated carbohydrate antigens), the agent inhibiting the metastasis potential of the preparation. Suitable biologic preparations include cell cultures and biologic fluids.

Another aspect of the instant invention provides a method for inhibiting metastasis potential of tumor cells or inflammation in a warm-blooded animal. The method comprises administering to a warm-blooded animal an effective amount of at least one agent selected from the group consisting of (a) tumor-associated carbohydrate antigens (or leukocyte-associated carbohydrate antigens) that exhibit differential prognostic significance, (b) antibodies that specifically bind to those antigens, (c) oligosaccharide components of those antigens, (d) conjugates of those antigens or oligosaccharide components and (e) mimetics of the tumor-associated carbohydrate antigens (or leukocyte-associated carbohydrate antigens), the agent inhibiting tumor cell metastasis potential or inflammation potential.

Within a related aspect, th instant invention provides a vari ty of glycoconjugates useful for prolonging the in vivo half-life of oligosaccharide comp nents. The conjugates compris an olig saccharide c upled to polyethyleneglyc l.

Additional oligosaccharide components for use within the methods and compositions of the instant invention include lactose, lacto-N-tetrose, methyl β-D-lactoside and phenyl β-D-thiolactoside. Oligosaccharide components may be used individually or in combination with one another.

The instant invention further provides a variety of methods for inhibiting GMP-140-mediated or ELAM-1-mediated cell aggregation or adhesion causing metastasis at a tumor site and inflammatory responses at a site.

One such method inhibits GMP-140-mediated or ELAM-1-mediated cell aggregation or adhesion within a biologic preparation and comprises incubating the biologic preparation with at least one agent selected from the group consisting of: (a) a hybrid sugar molecule, such as one comprising Le^x and SLe^x (Structure 1 of Figure 20, a branched type II chain); (b) a mixture of the components of the hybrid sugar of (a), such as, Le^x and SLe^x; (c) monosialosyl-Le^a I, Le^a, Le^x, monosialosyl-Le^a II, disialosyl-Le^a or sialosyl Le^x; (d) antibodies that specifically bind to a hybrid sugar, such as Le^x/SLe^x, or to the components thereof; (e) antibodies that specifically bind to monosialosyl-Le^a I, Le^a, Le^x, monosialosyl-Le^a II, disialosyl-Le^a or sialosyl Le^x; (f) oligosaccharide components of hybrid sugars, such as Le^x/SLe^x, monosialosyl-Le^a I, Le^a, Le^x, monosialosyl-Le^a II, disialosyl-Le^a r sialosyl Le^x; (g)

conjugat s of hybrid sugars, such as SLe^x/Le^x, monosialosyl-Le^a I, Le^a, Le^x, monosialosyl-Le^a II, disialosyl-Le^a or sialosyl Le^x or of the oligosaccharide components; and (h) mimetics of hybrid sugars, such as Le^x/SLe^x, monosialosyl-Le^a I, Le^a, Le^x, monosialosyl-Le^a II, disialosyl-Le^a or sialosyl Le^x, said agent inhibiting the cell aggregation or adhesion.

Another such method inhibits GMP-140-mediated or ELAM-1-mediated cell aggregation or adhesion at a tumor cell or inflammatory site in a warm-blooded animal thereby reducing metastatic potential or inflammation at the site and comprises administering to the warm-blooded animal an effective amount of at least one agent selected from the group consisting of: (a) a hybrid sugar, such as, SLe^x/Le^x; (b) a mixture of the components of a hybrid sugar (a), such as, Le^x and SLe^x; (c) monosialosyl-Le^a I, Le^a, Le^x, monosialosyl-Le^a II, disialosyl-Le^a or sialosyl Le^x; (d) antibodies that specifically bind to a hybrid sugar, such as Le^x/SLe^x, monosialosyl-Le^a I, Le^a, Le^x, monosialosyl-Le^a II, disialosyl-Le^a or sialosyl Le^x; (e) a mixture of antibodies, particularly to the components of a hybrid sugar, such as to Le^x, SLe^x, Le^a or SLe^a; (f) oligosaccharid components of a hybrid sugar, such as Le^x/SLe^x, monosialosyl-Le^a I, Le^a, Le^x, monosialosyl-Le^a II, disialosyl-Le^a or sialosyl Le^x; (g) conjugates of a hybrid sugar, such as, Le^x/SLe^x, monosialosyl-Le^a I, Le^a, Le^x, monosialosyl-Le^a II, disialosyl-Le^a or sialosyl Le^x or of the oligosaccharide components; and (h) mimetics of a hybrid sugar, such as, Le^x/SLe^x, monosialosyl-Le^a I, Le^a, Le^x, monosialosyl-Le^a II, disialosyl-Le^a

r sialosyl Le^x, the agent reducing the m tastatic potential at the tumor cell site or inflammation in th warm-blo ded animal.

The instant invention also provides a method f inhibiting GMP-140-mediated or ELAM-1-mediated cell aggregation or adhesion at a site of inflammation in a warm-blooded animal thereby reducing inflammatory potential at the site and comprises administering to warm-blooded animal an effective amount of at least one agent selected from the group consisting of (a) a hybrid sugar, such as, Le^x/SLe^x; (b) an appropriate mixture of sugars which are the components of a hybrid sugar (a), such as, Le^x and SLe^x; (c) monosialosyl-Le^a I, Le^a, Le^x, monosialosyl-Le^a II, disialosyl-Le^a or sialosyl Le^x; (d) antibodies that specifically bind to a hybrid sugar, such as, Le^x/SLe^x, monosialosyl-Le^a I, Le^a, Le^x, monosialosyl-Le^a II, disialosyl-Le^a or sialosyl Le^x; (e) a mixture of antibodies, particularly to the components of a hybrid sugar, such as to Le^x, SLe^x, Le^a and SLe^a; (f) oligosaccharide components of a hybrid sugar, such as, SLe^x/Le^x, monosialosyl-Le^a I, Le^a, Le^x, monosialosyl-Le^a II, disialosyl-Le^a or sialosyl Le^x; (g) conjugates of a hybrid sugar, such as SLe^x/Le^x, monosialosyl-Le^a I, Le^a Le^x, monosialosyl-Le^a II, disialosyl-Le^a or sialosyl Le^x or of the oligosaccharide components; and (h) mimetics of a hybrid sugar, such as, Le^x/SLe^x, monosialosyl-Le^a I, Le^a, Le^x, monosialosyl-Le^a II, disialosyl-Le^a or sialosyl Le^x, the agent reducing the inflammatory potential at the inflammatory site in the warm-blooded animal.

In another aspect, the instant invention provides a method f r identifying a tumor associated carbohydrate antigen (TACA)

epitope to which lectin activity of GMP-140 is directed, comprising: (A) constructing a fluorescent probe comprising fluorescent plastic beads coated with the TACA epit pe susp cted of being targeted by GMP-140; (B) incubating the fluorescent probe with a suspension of platelets; and (C) determining the degree of binding of the fluorescent probe to the platelets.

Those and other aspects of the instant invention will become evident on reference to the following detailed description and attached drawings.

Brief Description of the Drawings

Figure 1 graphically illustrates the effects of methyl β-D-lactoside or methyl β-D-thiolactoside on the number and size of lung colony deposits of BL6 cells. BL6 cells were preincubated with control medium, 0.1 M methyl β-D-lactoside ("Me-β-lactoside") or 0.1 M phenyl β-D-thiolactoside ("phe-β-S-lactoside). Twenty thousand cells were injected intravenously into C57Bl mice. Lung colony numbers were counted at 21 days and colonies were classified on the basis of diameter (> 1 mm vs. < 1 mm), as indicated for each bar. Colony numbers are expressed per single lung. Number of experiments ("n") is indicated in parentheses.

Figure 2 graphically illustrates the effect of prior administration of methyl β-D-lactoside on the number and size of lung colony deposits of BL6 cells. Methyl β-D-lactoside (1 ml dose) was injected intraperit neally into C57Bl mice. After 10 minutes, BL6 melanoma cells were injected intravenously. Lung

colonies w re counted and sized at 19 days. Group A represents control animals (not administered with methyl β-D-lactoside) and gr ups B and C represent animals inject d with 0.25 M and 0.5 M methyl β-D-lactoside, respectively. For each group, column 1 represents the total number of colonies, column 2 the number of colonies with diameter > 1 mm and column 3 the number of colonies with diameter < 1 mm. Number of experiments is expressed as "n".

Figure 3 graphically illustrates survival of cancer patients with or without expression of a defined tumor-associated carbohydrate antigen (TACA) in the tumors. Panel 3A represents the expression of $H/Le^y/Le^b$ antigen in lung squamous cell carcinoma as determined by monoclonal antibody MIA-15-5. Panel 3B represents sialosyl-Le^x expression in colonic canc r using antibody FH6. Panel 3C represents sialosyl-Tn expression in colonic cancer using antibody TKH2. Panel 3D represents sialosyl-Tn level in sera of ovarian cancer patients.

Figure 4 graphically illustrates that melanoma cell adhesion on LacCer is based on GM3-LacCer interaction. The order of metastatic potential is BL6>F10>F1>>Wa4. Panel 4A shows the order of melanoma cell adhesion on a LacCer-coated solid phase. Panel 4B shows the order of melanoma cell adhesion on LacCer/Fibronectin (FN) co-coated solid phase. Panel 4C shows integrin-dependent adhesion.

Figure 5 graphically illustrates the melanoma cell (BL6) adhesion on LacCer (Panel 5A) and on endothelial cells (HuVEC) (Panel 5B) is inhibited by LacCer and GM3.

Figure 6 graphically illustrates the metastasis-inhibiting effect of methyl(Me)-β-lactoside. Tum r cells were injected

intravenously, followed by intraperitoneal injection of: PBS (A); 0.25 M Me-β-lactoside (B); 0.5 M Me-β-lactoside (C); 0.5 M lactose (D); 0.25 M N-ac tyllactosamine (E); or 0.5 M Me-β-galactoside (F).

Figure 7 graphically illustrates H-Le^Y and H-H interaction. Panel 7A shows H1-liposome binding to various glycolipids. Panel 7B shows Le^Y-liposome binding to various glycolipids.

Figures 8A-8D are flow cytometric profiles of non-activated (Panels 8A and 8C) and activated (Panels 8B and 8D) platelets with anti-GMP-140 monoclonal antibody.

Figure 9 graphically illustrates the binding indices of platelets with fluorescent beads coated with various GSL's. The hatched bars represent non-activated platelets and the open bars represent activated platelets.

Figure 10 graphically illustrates the effects of various monoclonal antibodies on binding of activated platelets to sialosyl-Le^a-coated beads. The abscissa represents the percent inhibition. Column 1 represents anti-GMP-140-mAb, IOP62; column 2 represents anti-sialosyl-Le^a monoclonal antibody, CA19-9; column 3 represents anti-sialosyl-Le^x monoclonal antibody, SNH4; and column 4 represents normal mouse IgG.

Figures 11A-11D illustrate experimental systems demonstrating dynamic adhesion of cells in a flow system. Panel 11A shows the structure of the laminar flow chamber. Panel 11B depicts a cross section of a laminar chamber in which the flow chamber body (16) is affixed tightly with the cover slip (3) on which cells or adhesion molecules (9) are fixed. Panel 11C shows the entire assembly of the recording system.

Panel 11D is a schematic pres ntation of the flow of tumor cells in suspension passing over th cell lay r or adhesion molecules.

Figure 12 is a graph showing the effect f various monoclonal antibodies on adhesion of human c lon carcinoma Colo205 cells to interleukin-1-activated human umbilical vein endothelial cells in a dynamic flow system. Open circl s represent a mixture of irrelevant mouse IgG plus IgM (control), the solid triangles represent monoclonal antibody CA19-9 direct d to monosialosyl-Le^a I, the open triangles represent monoclonal antibody SNH4 directed to sialosyl-Le^x, the solid circles represent monoclonal antibody FH7 directed to monosialosyl-Le^a II and disialosyl-Le^a and the solid squares represent a mixture of irrelevant mouse IgG plus IgM and non-activated endothelial cells.

Figure 13 depicts binding of mAb's to HL60 cells and th effect of sialidase thereon. Binding activity was determined by flow cytometry. Abscissa: log fluorescence intensity. Ordinate: relative cell number. Panel A: Solid line, cells stained with mAb SNH4 as primary antibody. Dotted line, control cells stained with mouse IgG plus IgM [10 μg/ml] as primary antibody. Panel B: mAb SNH3 as primary antibody; control as in Pane A. Panel C: Solid line, cells treated with Newcastl Disease Virus (NDV) sialidase and then stained with mAb SNH4. Dotted line, control cells (as in Panel A, after sialidas treatment). Panel D: NDV sialidase followed by mAb SNH3; control as in Panel C. Panel E: Vibrio cholerae (VC) sialidase followed by mAb SNH4. Panel F: VC sialidase followed by mAb

SNH3. Note that expression of SLe^x (defined by both SNH3 and SNH4) was abolished completely by both NDV and VC sialidases.

Figure 14 depicts adhesion of HL60 cells to E-selectin-coated plates in a static system. Abscissa, type of treatment. Ordinate, percent cell adhesion relative to untreated control cells. Panel A: effects of various sialidases. Panel B: effects of anti-Le^x and anti-SLe^x mAb's alone and in combination (incubated 90 min at 37°C). Panel C: effects of NDV sialidase plus mAb. Panel A: NDV sialidase (which cleaves $\alpha 2 \rightarrow 3$ sialosyl at a terminal Gal, eliminates the SLe^x structure and abolishes reactivity with mAb's SNH3 and SNH4, see Figure 13, but did not abolish adhesion. VC and Arthrobacter ureafaciens (AV) sialidases did abolish adhesion. Panel B: anti-SLe^x mAb's were less effective than anti-Le^x mAb's. Combinations of both types of mAb's were most effective. Panel C: Adhesion was inhibited most effectively by NDV sialidase plus anti-Le^x mAb.

Figure 15 depicts adhesion of HL60 cells to E-selectin-coated plates in a dynamic flow system. Truncated E-selectin was coated onto marked areas (diameter of about 0.5 cm) on plastic plates and adhesion under defined wall shear stresses was assayed as described herein. Abscissa, shear stress (dynes/cm^2). Ordinate, number of cells adhered within 3 min. Panel A: hollow circle, control (untreated) cells; solid triangle, cells treated with NDV sialidase; solid circle, VC sialidase; and hollow triangle, AU sialidase. Panel B: hollow circle, control; solid triangle, cells cultured in medium containing anti-SLe^x IgG_3 mAb SNH4; solid circle, anti-Le^x IgM mAb FH2; and hollow triangle, anti-Le^x IgG_3 mAb SH1.

Panel C: hollow circle, control; solid triangle, NDV sialidase; solid circl , mAb SH1; and hollow triangl , NDV sialidase plus mAb SH1. Pan l D: holl w circle, control; solid circle, mixture (1:1) of mAb's SNH4 and FH2; and hollow triangle, mixture (1:1) of mAb's SNH4 and SH1. Cleavage of α2→3 sialosylation at a terminal Gal by NDV sialidase reduced adhesion somewhat, however adhesion remained at low shear stress. In contrast, VC and AU sialidases strongly inhibited adhesion (Panel 15A) indicating the importance of internal sialosylation (which is unaffected by NDV sialidase). That observation is substantiated by observations that (i) NDV sialidase plus mAb SH1 strongly inhibited adhesion and (ii) combination of anti-SLe^x mAb SNH4 plus anti-Le^x mAb's FH2 or SH1 inhibited adhesion more strongly than SNH4 alon (Panels 15B and 15D).

Figure 16 depicts reactivity of Colo201 cells with various mAb's, with or without sialidase treatment. Colo201 cells were reactive strongly with anti-SLe^a I mAb's CA19-9 and NKH1 (Panel A), anti-Le^a mAb CA3F4 (Panel B) and anti-SLe^a II mAb FH7 (Panel C). Reactivity with CA19-9 was decreased by NDV sialidase (Panel D) and abolished by VC sialidase (Panel G). Reactivity with CA3F4 was increased slightly by NDV and VC sialidas s (Panels E and H). Reactivity with FH7 was unchanged by NDV sialidase (Panel F) and decreased slightly by VC sialidase (Panel I).

Figure 17 depicts adhesion of Colo201 cells to E-selectin-coated plates in a static system. Abscissa and ordinate as in Figure 14. Panel A: effects of various sialidas s (90 min. incubation, 37°C). Panel B: ff cts of

sialidases (18 hr. incubation, 37°C), cells were first fixed with 0.5% paraformaldehyde for 10 minutes at room temperature. Panel C: effects f sialidases followed by mAb's. NDV sialidase, which cleaves α2→3 sialosyl at terminal Gal, did not affect adhesion, whereas VC and AU sialidases, which cleave sialic acid residues regardless of location, abolished adhesion (Panel B). In Panel C, most effective inhibition was observed with VC or AU sialidase plus mAb CA3F4.

Figure 18 depicts adhesion of Colo201 cells to E-selectin-coated plates in a dynamic flow system. The adhesion assay is as described herein. Abscissa and ordinate as in Figure 15. Panel A: hollow circle, control; solid circle, NDV sialidase; hollow triangle, AU sialidase; and solid triangle, VC sialidase. Panel B: hollow circle, control; solid circle, anti-SLe^a I mAb CA19-9; hollow triangle, anti-SLe^a II mAb FH7; and solid triangle, anti-Le^a mAb CA3F4. Panel C: hollow circle, control; solid circle, CA3F4; solid triangle, VC sialidas ; hollow inverted triangle, VC sialidase plus CA19-9; and hollow triangle, VC sialidase plus CA3F4 (note that adhesion was most strongly inhibited by that combination). Panel D: hollow circle, control; solid triangle, NDV sialidase; solid inverted triangle, CA3F4; hollow inverted triangle, NDV sialidase plus CA19-9; solid circle, NDV sialidase plus FH7; and hollow triangle, NDV sialidase plus CA3F4 (note that adhesion was inhibited most strongly by that combination).

Figure 19 depicts the effect of Newcastle Disease Virus (NDV) sialidase, Vibrio cholerae (VC) sialidase or mAb's SNH4 or SH1 on HL60 binding to ELAM-coated plates in a dynamic flow

system under various shear strength conditions. The ordinate represents per cent cell binding relativ to untr ated control cells. The antibodies were used at 15 μg/ml, NDV sialidase at 0.2 U/ml and VC sialidase at 0.1 U/ml. Each point represents th mean of three experiments. Number of untreated cells bound at shear stresses of 15.5, 7.75, 3.13, 1.56 and 0.78 dynes/cm^2 were 4.5, 27, 109.6 206.2 and 283.8 cells/mm^2, respectively.

Figure 20 depicts various branched sugars. The hybrid sugar, Le^x/SLe^x, is depicted as structure 1. The glycolipids containing such a structure were isolated from colon carcinoma or were prepared from G8 ganglioside presented in Structure 5 originally found in human erythrocytes (Watanabe et al., J. Biol. Chem., 254:8223, 1979) by enzyme catalyzed α1→3 fucosylation. Structure 2 was obtained by α1→3 fucosylation of compound 6 originally obtained from human placenta. Structure 2 however did not exhibit high affinity binding to E-selectin. Structures 3 and 4 depict analogs with high affinity binding sites having Le^x and sialyl-Galβ1→3GalNac within the same molecule (Structure 3), or the hybrid molecule Le^a/SLe^a, the positional isomer of structure 1.

Figure 21 depicts the relative adhesion of NS-1 cells expressing E-selectin on various "glyco-liposomes" coated on a plastic surface. Panel 21A shows the result of such relative adhesion in a dynamic flow setting under middle shear stress conditions (7.75 dynes/cm^2). The first seven bars indicate relative adhesion of NS-1 cells to SLe^x on each glycoliposome as indicated. Cpd I is structure 1 of Figure 20 and Cpd II is structur 2 of Figure 20. Bars 8-10 show a mixture of Le^x with

different types of compounds as indicated. The value of relative adhesion is expressed in comparison with the adhesion of SLe^x-liposome as 100%. Values represent the mean of five determinations. Panel 21B indicates the same relative adhesion of NS-1 cells at high shear stress conditions (11.8 dynes/cm^2). The value is expressed in terms of the adhesion on SLe^x-coated plates. Values represent the mean of five determinations.

Figure 22 depicts the relative adhesion of NS-1 cells expressing E-selectin on various glycoliposomes coated on plastic plates at different shear stress conditions. CPD I and CPD II are structures 1 and 2 of Figure 20. Enhancement of adhesion on CPD I-coated plates was noted only at middle to high shear stress conditions. The ordinate indicates the relative adhesion as compared with that of the SLe^x liposome. The abscissa indicates the wall shear stress in dynamic flow in dynes/cm^2. DSI represents disialosyl-I antigen.

Figure 23 depicts cell numbers bound per square millimeter on various glycoliposomes coated on a plastic surface with different glycolipid concentrations. Note that structure 1 of Figure 20 adheres E-selectin-expressing cells much more avidly than on SLe^x-coated plates at high shear stress. The difference is not as stark at low shear stress. The ordinate indicates the number of cells bound per millimeter and the abscissa indicates glycolipid concentration in μm. Each point is the mean of five determinations.

Figure 24 depicts adhesion of NS-1 cells expressing E-selectin on glycoliposomes having a mixture of SLe^x and various other glycolipids. The ordinate shows the number of cells

adhered p r fi ld. The solid circle is SLe^x + SPG. Th hollow circle is SLe^x + H2. The solid triangl is SLe^x + Le^x. The hollow triangle is SLe^x + Le^y. Each point is the mean of five determinati ns.

Detailed Description of the Invention

As noted above, the instant invention in one aspect is directed to methods and compositions for the inhibition of tumor cell metastasis potential and invasiveness. Numerous tumor cells possess the ability to metastasize, i.e., to form a secondary tumor colony at a distant site. Sources of malignant tumor cells include melanoma, lung, breast, colorectal and urogenital cancers, such as bladder and prostate cancers. Within the instant invention, the metastasis potential of tumor cells, (i.e., the ability of tumor cells to metastasize) may b inhibited through the use of (a) tumor-associated carbohydrate antigens (TACA's, as used herein TACA is meant to include LACA); (b) antibodies directed to those TACA's; (c) oligosaccharide components of those TACA's; (d) conjugates of such TACA's or of oligosaccharide components of such TACA's, such as multivalent conjugates of lysyllysine or TACA-bearing glycosphingolipid (GSL) liposomes; or (e) mimetics of the TACA's. Generally, unless indicated to the contrary, tumor cells and leukocytes are substantial equivalents inasmuch as both bind to endothelial cells by carbohydrate structures.

TACA epitopes play essential roles in tumor cell adhesion through interaction with endothelial cells, platelets and basement membranes, whereby tumor metastasis and invasion may

ccur. The mechanism of adhesi n may be based on carbohydrat (CHO) CHO-CHO interaction, CHO-lectin int raction or CHO-selectin family interaction.

Adhesion of various tumor cells to non-activated endothelial cells is mediated initially by carbohydrate to carbohydrate interactions, which in turn, trigger activation of endothelial cells to express selectins, such as ELAM-1 and GMP-140, Kojima & Hakomori, J. Biol. Chem., 266:17552, 1991; Kojima et al., J. Biol. Chem., 267:17264, 1992; Hakomori, Histochem. J., 24:771, 1992. Subsequently, adhesion of various tumor cells to activated endothelial cells and platelets is mediated primarily by the LECCAM or selectin superfamily (e.g., ELAM-1 and GMP-140).

Tumor cell adhesion mediated by sialosyl-Le^x is inhibited by anti-sialosyl-Le^x monoclonal antibodies (FH6, CSLEX, SNH3 and SNH4) and tumor cell adhesion mediated by monosialosyl-Le^a I is inhibited by monoclonal antibodies (CA19-9, CSLEA, NKH1 and NKH2) directed to that epitope. Handa et al., Biochem. Biophys. Res. Commun. 181:1223, 1991; Kojima et al., Biochem. Biophys. Res. Commun. 182:1288, 1992; Hakomori, Histochem. J., 24:771, 1992.

The adhesion of Colo205 tumor cells, which express predominantly type 1 chain sialosyl-Le^a and to a lesser extent sialosyl-Le^x, to endothelial cells is inhibited by anti-sialosyl-Le^a monoclonal antibody and to a lesser extent by anti-sialosyl-Le^x monoclonal antibody. Those findings suggest that not only sialosyl-Le^x, but also sialosyl-Le^a, are the important ligands recognized by ELAM-1 and GMP-140 (previously termed CD62 or PADGEM and also known as E-selectin and P-selectin).

It is kn wn now that adhesion f tumor cells to activated ndothelial cells is bas d also on recognition of monosialosyl-Le^a II and disialosyl-Le^a. Both monosialosyl-Le^a II and disialosyl-Le^a are defined by monoclonal antibody FH7, which is known to inhibit strongly adhesion of various types of epithelial cancer cells (particularly colorectal, gastrointestinal and pancreatic) to activated endothelial cells or platelets via selectins.

In particular, GMP-140 is the major selectin (LECCAM) located on α-granules of platelets or Weibel-Pallade bodies of endothelial cells (EC's). On activation of those cells, GMP-140 is redistributed rapidly to the cell surface, where it plays an important role in adhesion of platelets or EC's to certain carbohydrate epitopes expressed on blood cells or tumor cells, resulting in aggregation of platelets or tumor cells, or adhesion thereof to capillary endothelia. GMP-140-mediated cell adhesion is believed by the instant inventors to be involved in initiation of metastatic deposition of tumor cells and initiation of inflammatory processes.

Also, ELAM-1 is expressed on endothelial cells after activation with interleukin-1, TGF-β, TNF-α or lipopolysaccharide. ELAM-1-mediated cell adhesion also is believed to be involved in initiation of metastatic deposition of tumor cells.

Thus, the instant invention in another aspect is directed to inhibiting GMP-140-mediated or ELAM-1-mediated cell aggregation or adhesion, especially at tumor cell sites. Within th instant invention, GMP-140-mediat d or ELAM-1-m diated cell

aggregation or adhesion can be inhibited through the use of (a) a hybrid sugar, such as Le^x/SLe^x; (b) a mixture of sugars which are the components of a hybrid sugar (a), such as Le^x and SLe^x; (c) monosialosyl-Le^a I, Le^a, Le^x, monosial syl-Le^a II, disialosyl-Le^a or sialosyl Le^x; (d) antibodies that specifically bind to a hybrid sugar (a), such as Le^x/SLe^x, monosialosyl-Le^a I, Le^a, Le^x, monosialosyl-Le^a II, disialosyl-Le^a or sialosyl Le^x; (e) a mixture of antibodies, particularly to the components of a hybrid sugar, such as to SLe^x and Le^x; (f) oligosaccharide components of a hybrid sugar, such as Le^x/SLe^x, monosialosyl-Le^a I, Le^a, Le^x, monosialosyl-Le^a II, disialosyl-Le^a or sialosyl Le^x; (g) conjugates of a hybrid sugar, such as Le^x/SLe^x, monosialosyl-Le^a I, Le^a, Le^x, monosialosyl-Le^a II, disialosyl-Le^a or sialosyl Le^x or of the oligosaccharide components; and (h) mimetics of a hybrid sugar, such as Le^x/SLe^x, monosialosyl-Le^a I, Le^a, Le^x, monosialosyl-Le^a II, disialosyl-Le^a or sialosyl Le^x.

Within the instant invention, tumor metastasis and invasion is inhibited by blocking tumor cell adhesion thereby significantly reducing or eliminating the spread of metastatic cells.

Also within the instant invention, tumor metastasis and invasion is minimized by inhibiting: (1) GMP-140-mediated tumor cell aggregation or adhesion at a tumor site due to: (a) adhesion of tumor cells to platelets, (b) adhesion of tumor cells to tumor cells via platelets, (c) adhesion of tumor cells to EC's via platelets and (d) adhesion of tumor cells to EC's directly via GMP-140; and (2) ELAM-1-mediated tumor cell aggregation r

adh sion at a tumor site due to adhesi n of cells to EC's dir ctly via ELAM-1.

Further within th instant invention, inflammation is minimized by inhibiting GMP-140-mediat d leukocyte aggregation, adhesion or migration at a potential site of inflammation due to: (a) adhesion of leukocytes to platelets, (b) adhesion of leukocytes to endothelial cells (EC) via platelets, (c) adhesion of leukocytes to EC's directly via selectin and (d) transendothelial migration of leukocytes.

TACA's suitable for use within the instant invention are those showing differential prognostic significance (i.e., TACA's that may be correlated clearly with invasive or metastatic potential). Within the context of the instant invention, such TACA's may be distinguished through a comparison of invasiveness, metastasis and clinical prognosis of similar tumors showing expression vs. non-expression of such TACA's. Preferred TACA's for use within the present invention include H/Le^y/Le^b, sialosyl-Le^x (SA-Le^x or SLe^x), Le^a, Le^x, monosialosyl-Le^a I (SLe^a or SA-Le^a) and sialosyl-Tn (SA-Tn or STn). Derivatives of such TACA's include hybrid sugars, such as Le^x/SLe^x, dimeric Le^x, sialosyl-dimeric Le^x, trifuscosyl Le^y, disialosyl-Le^a and monosialosyl-Le^a II.

As noted above, TACA's for use within the instant invention exhibit a differential prognostic significance. By way of example, such a differential prognostic significance may be illustrated by the fact that tumors expressing H/Le^y/Le^b antigens (as defined by monoclonal antibody MIA-15-5) showed much wors patient prognosis than tumors not expressing thos antig ns. For

instance, as shown in Figure 3A, patients with squamous cell lung carcinoma expressing H/Le^y/Le^b had only an 11% survival over a 5-year period (i.e., 89% di d) whereas comparable patients not expressing H/Le^y/Le^b had an approximately 62% survival over the same period.

Similar results were obtained for tumors showing expression vs. non-expression of sialosyl-Le^x and sialosyl-Tn antigens. More specifically, as shown in Figure 3B, patients with colonic cancer expressing sialosyl-Le^x had only a 15% survival over a 5-year period, whereas comparable patients not expressing that antigen had an approximately 50% survival over that period. In a separate study, the 5-year survival of patients with early-stage colonic cancer not expressing sialosyl-Tn was 100%, as compared to 75% for patients who expressed sialosyl-Tn (se Figure 3C). As shown in Figure 3D, similar but more obvious differences were observed in patients with ovarian cancer showing expression vs. non-expression of sialosyl-Tn antigen.

Also as noted above, antibodies or a mixture of antibodies to suitable TACA's may be employed within the context of the instant invention. As used herein, such antibodies include both monoclonal and polyclonal antibodies and may be intact molecules, a fragment of such a molecule or a functional equivalent thereof. The antibody may be engineered genetically. Examples of antibody fragments include F(ab')$_2$, Fab', Fab and Fv.

Briefly, polyclonal antibodies may be produced by immunization of an animal and subsequent collection of sera therefrom. Immunization is accomplished, for example, by a systemic administration, such as by subcutaneous, intraspl nic

or intramuscular injection, into a rabbit, rat or mouse. It is preferred gen rally to follow th initial immunization with one or more booster immunizations prior t s ra c llecti n. Such methodology is well known and described in a number of references.

While polyclonal antibodies may be employed in the present invention, monoclonal antibodies are preferred. Monoclonal antibodies suitable for use within the instant invention includ those of murine or human origin, or chimeric antibodies such as those which combine portions of both human and murine antibodies (i.e., antigen binding region of murine antibody plus constant regions of human antibody). Human and chimeric antibodies may be produced using methods known by those skilled in the art. Human antibodies and chimeric human-mouse antibodies ar advantageous because such antibodies are less likely than murine antibodies to cause the production of anti-antibodies wh n administered clinically.

Monoclonal antibodies may be produced generally by th method of Köhler and Milstein (Nature 256:495, 1975; Eur. J. Immunol. 6:511, 1976), as well as by various techniques which modify the initial method of Köhler and Milstein (see Harlow and Lane (eds.), "Antibodies: A Laboratory Manual", Cold Spring Harbor Laboratory, 1988, which is herein incorporated by reference in its entirety).

Briefly, the lymph nodes and/or spleen of an animal immunized with one of the TACA's or the oligosaccharid components thereof are fused with myeloma cells to form hybrid cell lines ("hybridomas" or "clones"). Each hybridoma secretes

a single type of immunoglobulin and, like th myeloma cells, has the potential for ind finite cell division. It may be desirable to couple such molecules to a carrier to incr as immunogenicity. Suitabl carriers include keyhole limpet hemocyanin, thyroglobulin, bovine serum albumin and derivatives thereof.

An alternative to the production of monoclonal antibodies via hybridomas is the creation of monoclonal antibodies expression libraries using bacteriophage and bacteria (e.g., Sastry et al., Proc. Natl. Acad. Sci. USA 86:5728, 1989; Hus et al., Science 246:1275, 1289). Selection of antibodi s exhibiting appropriate specificity may be performed in a variety of ways which will be evident to those skilled in the art.

Representative examples of monoclonal antibodies suitable for use within the present invention include MIA-15-5 (Miyake & Hakomori, Biochem. 30:3328, 1991), as well as the monoclonal antibodies cited in Hakomori, Advances In Cancer Research 52:257-331, 1989.

As discussed above, oligosaccharide components of suitabl TACA's also may be used in the instant invention. As used herein, the term "oligosaccharide" includes naturally derived oligosaccharides, synthetically prepared and mimetic derivatives of either, including portions of a TACA oligosaccharide component.

Additional oligosaccharide components useful in the instant invention include lactose and lactose derivatives, such as methyl β-D-lactoside, lact-N-tetrose (Galβ1→3GlcNAcβ1→3Galβ1→4Glc) and phenyl β-D-thiolactoside. For example, both compounds were found to inhibit melanoma cell metastasis in the mouse lung. Other

lactose derivatives also may be used, including ethyl or phenyl lactosid and methyl or ethyl thi lactoside. It is believed that such lact s derivatives block binding of melanoma cells to EC's by inhibiting melanoma cell GM_3 gangli side interaction with lactosyl ceramide of the EC's.

Other oligosaccharide components suitable for inhibiting metastasis potential of cells of a particular tumor may be identified based on determining the structure of specific carbohydrate chain(s) which are involved in the ability of th tumor to metastasize. The identification of carbohydrate-containing molecules involved in the ability of a tumor to metastasize may be accomplished in a variety of ways, including through the use of glycosidases and inhibitors f glycosyltransferases.

The structure of carbohydrates bound to either lipids or proteins may be determined based on degradation, mass spectrometry, including electron-impact direct-probe (EI) and fast atom bombardment (FAB), and methylation analysis (techniqu s described, for example, in Nudelman et al., J. Biol. Chem. 261:5487, 1986). Degradation analysis may be accomplished chemically and/or enzymatically, e.g., by glycosidases. Th carbohydrate sequence suggested by degradation analysis may b determined by methylation analysis (Hakomori, J. Biochem. 55:205, 1964) followed by chemical ionization mass spectrometry of permethylated sugars (Stellner et al., Arch. Biochem. Biophys. 155:464, 1974; Levery et al., Meth. Enzymol. 138:13, 1987).

Alternatively, or in conjunction with those techniques, EI mass sp ctrometry may be performed on perm thylated glycans or

after the appropriate d gradation of intact glycans (Kannagi et al., J. Biol. Chem. 259:8444, 1984; Nudelman et al., J. Biol. Chem. 263:13942, 1988). Homogeneity of the carbohydrate sequence may be demonstrated based on various chemical and physical criteria, including proton NMR spectroscopy of intact or methylated glycans and FAB mass spectrometry. Once the carbohydrate sequence has been determined, it will be evident to those of ordinary skill in the art to select an appropriate oligosaccharide for inhibiting the metastasis potential of a tumor cell.

As briefly discussed above, conjugates of suitable TACA's or oligosaccharide components thereof, such as multivalent conjugates with lysyllysine or TACA-bearing glycosphingolipid (GSL) liposomes (or glyco-liposomes), also may be used in th instant invention.

The components of the conjugate may be coupled covalently to one another either directly or via a linker group. A direct reaction between components is possible when each possesses a substituent capable of reacting with the other. For example, a nucleophilic group, such as an amino or sulfhydryl group, on on component may be capable of reacting with a carbonyl-containing group, such as an anhydride or an acyl halide, or with an alkyl group containing a leaving group, e.g., a halide, on the other.

It may be desirable to couple covalently components via a linker group. A linker group can serve to increase the chemical reactivity of a substituent and thus increase the coupling efficiency. An increase in chemical reactivity also may facilitate the use of functional groups on components which would

not otherwise b possible. F r exampl , a carboxyl group may b activated. Activation f a carboxyl group includes formation of an "active ester", such as a succinimidyl ester. The term "active ester" is known to refer to esters which ar highly reactive in nucleophilic substitution reactions.

Alternatively, it may be desirable to produce conjugates in which the components are linked non-covalently. For example, on or more TACA's may be incorporated into the outer surface of glycosphingolipid (GSL) liposomes.

It may be desirable to increase the in vivo half life of an oligosaccharide. As disclosed in the instant invention, oligosaccharides may be coupled to (i.e., covalently bonded to) a straight-chain amphophilic polymer, such as polyethyleneglycol. A representative example of a method for producing an oligosaccharide-polyethyleneglycol conjugate is the reaction f an oligosaccharide, which has been derivatized to contain a succinimidyl group, with a polyethyleneglycol having a terminal amino group. The latter compound has a general formula f $NH_2-(CH_2CH_2-O)_n-CH_3$, where n typically averages 44.7 (i.e., molecular weight of about 2,000) to 112.9 (i.e., molecular weight of about 5,000).

Additionally, because the cell adhesion mediated by selectins, ELAM-1 or GMP-140, is based on recognition of sialylated and fucosylated lactoseries type 1 and type 2 chains by a lectin sequence domain present at the N-terminal region of the selectin molecules, any structure which may show mor effective blocking activity of the lectin domain than naturally ccurring epitopes are useful in the present invention. Such

unnatural synthetic compounds, term d "mimetics", of, f r example, sialosyl-Le^x or sialosyl-Le^a I or II, which mimic the surface structure f naturally ccurring epitopes but show better blocking activity of carbohydrate-dependent adhesion, can be considered.

Examples of useful mimetics include, but are not limited to, sialosyl-Le^x or monosialosyl-Le^a I or II having trifluoro-L-fucose, N-trifluoro-acetyl-glucosamine or a heterocyclic or aromatic ring structure having a sialic acid analog and fucose analog at the same distance and spacial configuration as those found in naturally occurring sialosyl-Le^x, monosialosyl-Le^a I and II, or the H/Le^y/Le^b structure having trifluoro-L-fucose, N-trifluoro-acetyl-glucosamine or sialosyl-Tn analogs containing N-trifluoro-acetyl-neuraminic acid.

Thus, a modified carbohydrate epitope, or any other "mimetic" mimicking the surface structure of a carbohydrate epitope, which blocks cell adhesion through tumor-associated carbohydrates more efficiently than a naturally occurring epitop is within the scope of the instant invention.

The inhibition of metastasis potential of tumor cells and GMP-140-mediated or ELAM-1-mediated cell aggregation or adhesion have a variety of in vitro and in vivo uses, e.g., treatment of isolated tumor cells or tumor-bearing hosts and treatment of disease processes involving GMP-140 or ELAM-1.

Regarding in vitro aspects, as noted above, the instant invention provides a method for inhibiting tumor cell metastasis potential within a biologic preparation. The m thod comprises incubating a biologic preparation with at least one agent

selected from the group consisting of (a) tumor-associated carbohydrat antigens that exhibit differ ntial prognostic significance, (b) antibodies that specifically bind to those antigens, (c) oligosaccharide components of those antigens, (d) conjugates of those antigens or oligosaccharide components and (e) mimetics of the tumor-associated carbohydrate antigens, the agent inhibiting the metastasis potential of the preparation.

Regarding further in vitro aspects, the instant invention also provides a method for inhibiting GMP-140-mediated or ELAM-1-mediated cell aggregation or adhesion in a biologic preparation. The method comprises incubating the biologic preparation with at least one agent selected from the group consisting of (a) a hybrid sugar, such as Le^x/SLe^x; (b) sugar components of a hybrid sugar (a), such as Le^x and SLe^x; (c) monosialosyl-Le^a I, Le^a, Le^x, monosialosyl-Le^a II, disialosyl-Le^a or sialosyl Le^x; (d) antibodies that specifically bind to a hybrid sugar, such as Le^x/SLe^x or to the compon nt sugars thereof, monosialosyl-Le^a I, Le^a, Le^x, monosialosyl-Le^a II, disialosyl-Le^a or sialosyl Le^x; (e) oligosaccharide components of a hybrid sugar, such as Le^x/SLe^x, monosialosyl-Le^a I, Le^a, Le^x, monosialosyl-Le^a II, disialosyl-Le^a or sialosyl Le^x; (f) conjugates of a hybrid sugar, such as Le^x/SLe^x, monosialosyl-Le^a I, Le^a, Le^x, monosialosyl-Le^a II, disialosyl-Le^a or sialosyl Le^x or of the oligosaccharide components; and (g) mimetics of a hybrid sugar, such as SLe^x/Le^x, monosialosyl-Le^a I, Le^a, Le^x, monosialosyl-Le^a II, disialosyl-Le^a or sialosyl Le^x, the agent inhibiting the cell aggregation or adh si n.

Suitable biologic preparations include cell cultures and cell suspensions in biologic fluids, such as blood, urine, lymph, synovial and cerebrospinal fluid. TACA's, ligosaccharides or conjugates ther of generally will be incubated at a final concentration of about 0.1 to 1 M, and typically at about 0.2 to 0.5 M. Incubation is performed typically for 5 to 15 minutes at 37°C. After treatment of a biologic preparation, the preparati n may be injected or implanted in an animal, e.g., to confirm effectiveness of the inhibition of metastasis potential.

The instant invention also provides a method for inhibiting tumor cell metastasis potential in a warm-blooded animal, such as a human. The method comprises administering to a warm-blooded animal an effective amount of at least one agent selected from the group consisting of (a) tumor-associated carbohydrate antigens that exhibit differential prognostic significanc , (b) antibodies that specifically bind to those antigens, (c) oligosaccharide components of those antigens, (d) conjugates of those antigens or the oligosaccharide components and (e) mimetics of monosialosyl-Le^a I, Le^a, Le^x, monosialosyl-Le^a II, disialosyl-Le^a or sialosyl Le^x, the agent inhibiting the metastasis potential of the preparation.

Similarly, the instant invention also provides a method for inhibiting GMP-140-mediated or ELAM-1-mediated cell aggregation or adhesion at a tumor cell site in a warm-blooded animal. The method comprises administering to a warm-blooded animal an effective amount of at least one agent selected from the group consisting of (a) a hybrid sugar, such as Le^x/SLe^x; (b) component sugars of a hybrid sugar (a), such as Le^x and

SLe^x; (c) monosialosyl-Le^a I, Le^a, Le^x, monosialosyl-Le^a II, disialosyl-Le^a or sialosyl Le^x; (d) antibodies that sp cifically bind to a hybrid sugar, such as Le^x/SLe^x, monosialosyl-Le^a I, Le^a, Le^x, monosialosyl-Le^a II, disialosyl-Le^a or sialosyl Le^x; (e) oligosaccharide components of a hybrid sugar, such as Le^x/SLe^x, monosialosyl-Le^a I, monosialosyl-Le^a II, Le^a, Le^x, disialosyl-Le^a or sialosyl Le^x; (f) conjugates of a hybrid sugar, such as Le^x/SLe^x, monosialosyl-Le^a I, Le^a, Le^x, monosialosyl-Le^a II, disialosyl-Le^a or sialosyl Le^x or of th oligosaccharide components; and (g) mimetics of a hybrid sugar, such as Le^x/SLe^x, monosialosyl-Le^a I, Le^a, Le^x, monosialosyl-Le^a II, disialosyl-Le^a or sialosyl Le^x, said agent reducing the metastatic potential at the tumor site in the warm-blooded animal.

The instant invention also provides a method for inhibiting GMP-140-mediated cell aggregation or adhesion at an inflammati n site in a warm-blooded animal.

The method comprises administering to warm-blooded animal an effective amount of at least one agent selected from the group consisting of: (a) a hybrid sugar, such as Le^x/SLe^x; (b) component sugars of a hybrid sugar (a), such as Le^x and SLe^x; (c) monosialosyl-Le^a I, Le^a, Le^x, monosialosyl-Le^a II, disialosyl-Le^a or sialosyl Le^x; (d) antibodies that specifically bind to a hybrid sugar, such as Le^x/SLe^x, monosialosyl-Le^a I, Le^a, Le^x, monosialosyl-Le^a II, disialosyl-Le^a or sialosyl Le^x; (e) oligosaccharide components of a hybrid sugar, such as Le^x/SLe^x, monosial syl-Le^a I, Le^a, Le^x, monosialosyl-Le^a II, disialosyl-Le^a or sialosyl Le^x; (f) conjugates of a hybrid

sugar, such as SLe^x/Le^x, monosialosyl-Le^a I, Le^a, Le^x, monosialosyl-Le^a II, disialosyl-Le^a r sialosyl Le^x r f the olig saccharide components; and (g) mimetics of a hybrid sugar, such as Le^x/SLe^x, monosialosyl-Le^a I, Le^a, Le^x, monosialosyl-Le^a II, disialosyl-Le^a or sialosyl Le^x, the agent reducing the inflammatory potential at the inflammatory site in the warm-blooded animal.

For both methods, TACA's, oligosaccharides or conjugates thereof generally will be administered at a concentration of about 0.1 to 1 M and typically at about 0.2 to 0.5 M. It will be evident to those skilled in the art how to determine the optimal effective dose for a particular substance, e.g., based on in vitro and in vivo studies in non-human animals. A variety of routes of administration may be used. Typically, administration will be intravenous or intracavitary, e.g., in the pleural or peritoneal cavities, in the bed of a resected tumor or at a site of inflammation.

A TACA, antibody, oligosaccharide or derivative as discussed above may be administered in combination with a pharmaceutically acceptable carrier or diluent, such as physiologic saline. Moreover, the agents that inhibit or reduce metastatic potential may be administered in combination with an immunotherapeutic or chemotherapeutic substance, and the agents that reduce inflammatory potential may be administered in combination with an anti-inflammatory substance.

When a combination of such an agent and a substance is desired, each compound may be administered sequentially, simultaneously or combined and administered as a single

comp siti n. Diagnostic techniques, such as CAT scans, may be performed prior to and subsequent to administration t confirm the effectiveness of the inhibition of metastatic potential or inflammatory potential.

One in vitro system for measuring adhesion or aggregation of tumor cells to other cells (e.g. EC's), or for determining successful inhibition of adhesion or aggregation is a dynamic flow system similar to that described by M. B. Lawrence et al. (Blood 70:1284, 1987) and which is shown in Figures 11A, 11B, 11C and 11D.

A parallel-plate laminar flow chamber (1) (shown upside down for convenience) connected to a pressure pump (2) via tubing (18) is used to simulate the flow shear stresses present in physiological microvascular environments. The flow chamber consists of a plastic or glass cover slip (3) resting on a chamber body (16) on which a parallel, transparent plastic surface (4) is attached with a rubber or silicone gasket (5); there is a 114 μm gap between the two surfaces, and this gap is connected to an inlet slot (6) connected to an inlet manifold (8) and outlet slot (7) connected to an outlet manifold (19) (Figure 11A). A laminar flow with defined rate and wall sh ar stress is achieved by manipulation of the pressure pump (2), which is connected to the inlet manifold (8) of the flow chamber via tubing (18). Figure 11B depicts the configuration of an assembled flow chamber (1).

To fix together the cover slip (3) and the chamber body (16) very tightly, there is a continuous circular grooved space (20) on th periphery of th chamber body (16). Th circular, groov d

space connects to a vacuum pump by placement of the rubber or silicone rubber gasket (5) with cover slip (3) on top. Thus, by applying vacuum (21) in (20) the cover slip (3) and chamber body (16) are affixed strongly and immovable. The thin inl t and outlet slots in the chamber body (16) open to the inlet and outlet manifolds, respectively. The outlet manifold is connected to a pressure pump (2) which can be operated in either a negativ or positive mode.

Cells (e.g., endothelial cells) are grown on either a glass or plastic cover slip (3), or various adhesion molecules ar affixed on (3), and a tumor cell suspension in medium flows from inlet manifold (16) to outlet manifold (19). The structure of the flow chamber (1) in Figure 11B is shown upside down for convenience. The chamber is placed under an inverted microscope stage, right side up (Figure 11C), and the flow of tumor cells over the cell layer (e.g., endothelial cell layer) is observed under the microscope. The observed pattern of rolling and stopping (i.e., pattern of adhesion) of tumor cells can be recorded on videotape.

Turning to Figure 11C, the cells (9) are grown as a monolayer, or adhesion molecules are affixed, on the cover slip (3) and a laminar flow of tumor cell suspension (14), maintained in a vessel in a water bath (17), is passed through the chamb r via tubing (18). Cell movements are observed under an inverted phase-contrast microscope (10) and recorded by time-lapse videocassette recorder (11) using a video camera (12) and a digital image processor (13). Adhesion is observed as rolling followed by stopping of cells. Numb r of c lls bound during a

set time, e.g. 3 minutes, at different shear stresses, e.g., from 0.4 to 4.8 dynes/cm^2, are counted from s veral fields r corded on videotape (Figure 11B). Wall sh ar stress (T) is calculated as $3\mu Q/2ba^2$, where μ = coefficient f viscosity, e.g. 1.0 cP, Q = volumetric flow rate (cm^3/sec), a = half channel height, e.g. 5.7×10^{-3} cm, and b = channel width, e.g. 1.3 cm.

Figure 11D schematically shows laminar flow of tumor c ll suspension (14) through a chamber in which one surface is coat d with endothelial cells (9). Rolling or stopped cells (15) ar observed under an inverted microscope and recorded on videotap , as described above. The arrows indicate the direction of flow of the tumor cell suspension (14).

As mentioned above, the instant invention also provides a method for identifying a TACA epitope to which lectin activity of a selectin, such as GMP-140, is directed.

Previously, the TACA epitopes were studied based on th inhibitory effect of various glycosphingolipids (GSL's), GSL oligosaccharides or GSL-containing liposomes on adhesion of blood cells or tumor cells to a solid phase (e.g., a plastic surfac) coated with activated platelets. In practice, that meant coating a solid phase with gelatin, which was in turn was coated with activated platelets; platelets bind readily to a gelatin-coated solid phase via GpIIb/IIIa, the major platelet integrin receptor.

In studies using that method, binding of promyelocytic leukemia HL60 cells to platelet-coated solid phase was inhibited by liposomes containing a sialosyl-Le^x determinant, but not by liposomes containing sialosylparaglobosid (SPG), and only weakly by liposomes containing $\alpha 1 \rightarrow 3$ fucosylat d type 2 chain (Le^x)

(see Table 1 in Example 3 below). Thos results suggested that sialosyl-Le^x is th carbohydrate epitope defined by GMP-140 (Polley et al., Proc. Natl. Acad. Sci. USA 88: 6224, 1991).

However, th method described above had an important limitation: no cell line which expresses exclusively type 1 chain GSL is available. Myelogenous cell line HL60 and monocytic cell line U937 express exclusively type 2 chain and little, if any, type 1 chain. On the other hand, all known human tumor cell lines derived from colonic, gastric or lung carcinoma express both type 1 and type 2 chains. For those reasons, it is difficult to determine the real epitope to which GMP-140 binds. To address that problem, a new methodology was developed, as described below.

Fluorescent plastic (e.g. polystyrene) beads (diameter ≈ 0.5 μm) are coated with GSL. GSL's are known to be adsorbed strongly on such beads, which allows construction of fluorescent probes containing specific GSL's. Platelets (activated or non-activated) are incubated with such GSL-coated beads, followed by determination of platelet fluorescenc intensity by flow cytometry.

Using that method, activated platelets were found to show much stronger binding to fluorescent beads coated with monosialosyl-Le^a I (see Table 3) than to beads coated with any related GSL. The binding of platelets to sialosyl-Le^a-coated beads was inhibited by anti-GMP-140 monoclonal antibody or anti-sialosyl-Le^a monoclonal antibody, but not by anti-sialosyl-Le^x mon cl nal antibody. Although binding of activated platelets t sialosyl-Le^x-coated beads was observable,

the lev l of binding was much lower than binding to sialosyl-Le^a-coated beads. Th se r sults indicate that the primary pitope structure d fined by GMP-140 is sialosyl-Le^a, rather than sialosyl-Le^x.

Of course, other epitope structures defined by a selectin, such as GMP-140, can be identified using the instant inventiv method.

ELAM-1 (E-selectin) is expressed on the surface of activated endothelial cells. ELAM-1 has a carbohydrate-binding domain at the amino terminal region and indeed ELAM-1 is known to bind SLe^x and SLe^a.

Those conclusions were based on the observations that adhesion between tumor cells or leukocytes to activated human EC's was inhibited by SLe^x glycolipid or oligosaccharide, but not by other tested glycolipids or oligosaccharides available at that time (Phillips et al., Science 250: 1130, 1990). Later, th above-noted adhesion was found to be inhibited by SLe^a I, th positional isomer of SLe^x glycolipid, as well (Berg et al., J. Biol. Chem., 266:14869, 1991; Takada et al., Biochem. Biophys. Res. Commun., 189:713, 1991).

That adhesion reaction was claimed to be inhibited by IgG_3 anti-SLe^x mAb (Phillips et al., supra). However, there are oth r structural variants related to SLe^x and SLe^a together with mAb's directed to those variants and the reported studies are based on inhibition of selectin-dependent adhesion by assumed epitope structure(s).

The instant invention is a result of systematic studies on selectin-dependent adhesion under static and dynamic

circumstances. For example, the m thods employed include, (i) adhesion of tumor cells to IL-1-activated human umbilical c rd ndothelial cells (HUVEC); (ii) adhesion of tumor cells to E-selectin-coated solid supp rts, for exampl , by using recombinant ELAM-1; (iii) adhesion of fluorescent particulate solid supports coated with glycoliposomes with activated platelets or HUVEC's expressing P-selectin or E-selectin; and (iv) adhesion of NS-1 myeloma cells, transfected with E-selectin coding sequences and permanently expressing E-selectin onto plates coated with glycoliposomes.

Hence, the systems (i), (ii) and (iii) were employed to assess the effect on adhesion of various mAb's directed to SLe^x, SLe^a I, SLe^a II, Le^x, Le^a and related structures; combinations of such mAb's; sialidases with various substrate specificities; or combinations of various sialidases and mAb's. The method of (iv) was used to compare the intensity of adhesion under dynamic conditions.

Specifically, the instant invention relates to carbohydrates defined by formulae (I), (II) and (III) below which are characterized by internal sialosyl residues or a branched structure.

Formula (I) relates to a type 1 or extended type 1 chain with internal α2→6 sialosyl substitutions and an α1→4 fucosyl substitution.

```
           NeuAcα2               R2            R2
                ↓                 |             |
                6                 |             |
Galβ1→3GlcNAcβ1[→3Galβ1→3GlcNAcβ1→]n3Galβ1→            (I)
 |              4                               |
 R1             ↑                               R3
            Fucα1
```

In formula (I), R_1 is H or a sialic acid residue in $\alpha 2\rightarrow 3$ linkage; R_2 is H or a sialic acid residue in $\alpha 2\rightarrow 6$ linkage; n is qual to or greater than 0; and R_3 is H or a fucosyl residue in $\alpha 1\rightarrow 4$ linkage.

Formula (II) relates to a type 2 chain structure with internal sialosyl and fucosyl substitutions.

$$\begin{array}{ccccc}
 & \mathrm{NeuAc}\alpha 2 & & & \\
 & \downarrow & & & \\
 & 6 & R_2 & R_2 & R_2 \\
 & & \vert & \vert & \vert \\
\mathrm{Gal}\beta 1\rightarrow 4 & \mathrm{GlcNAc}\beta 1[\rightarrow 3 & \mathrm{Gal}\beta 1\rightarrow 4 & \mathrm{GlcNAc}\beta 1\rightarrow]_n 3 & \mathrm{Gal}\beta 1\rightarrow \\
\vert & 3 & & \vert & \\
R_5 & \uparrow & & R_4 & \\
 & \mathrm{Fuc}\alpha 1 & & &
\end{array} \qquad \text{(II)}$$

In formula (II), R_2 is as defined for formula I, R_4 is H or a fucosyl residue in $\alpha 1\rightarrow 3$ linkage and R_5 is H, a sialic acid residue in $\alpha 2\rightarrow 3$ linkage, NeuAc$\alpha 2\rightarrow 8$NeuAc in $\alpha 2\rightarrow 3$ linkage or $R_6\rightarrow$NeuAc in $\alpha 2\rightarrow 3$ linkage, wherein R_6 is one or more sugars other than a sialic acid residue and n is equal to or greater than 0.

Formula (III) relates to a type 2 chain structure which is a hybrid molecule comprising a branch wherein each branch comprises an epitope of a single carbohydrate antigen as disclosed herein. Hence, as used herein, a hybrid molecule does not necessarily comprise the entirety of the two component sugars that comprise the hybrid. Instead, the hybrid comprises th epitopes of the component sugars. Hence, referring to Figure 20, structure 1 comprises the epitopes of Le^x and SLe^x, however it will be noted that with reference to the diagrammatic structures of the various sugars set forth hereinbelow, not all of the Le^x or SLe^x molecules are found in the hybrid. As to the SLe^x portion of the hybrid, only th terminal galactos and glucosamine, tog ther with the attached fucosyl and sialic acid

residues, of the intact SLe^x molecule comprise the hybrid. Similarly, for Le^x, only the epitope generating terminal three sugar residues comprise the hybrid. As used herein, epitop is that p rtion of the sugar which interacts in the adhesion phenomenon.

$$\begin{matrix} R_{10}1\rightarrow 6 \\ R_{11}1\rightarrow 3 \end{matrix} R_8\!-\!R_9 \qquad \text{(III)}$$

In formula III, each of R_{10} and R_{11} comprises galactose, Galβ1→4GlcNAc or Galβ1→3GlcNAc; R_8 comprises Gal or GalNAc; and R_9 comprises lactosyl ceramide or an O-linked sugar. Additionally, R_{10} and R_{11} may comprise fucosyl and sialic acid residues. The hybrid structures are identified by the respective epitopes contained therein. Hence, structure 1 of Figure 20 is denoted SLe^x/Le^x, or Le^x/SLe^x.

Formula I is based on inhibition by various mAb's and sialidases and combinations thereof of E-selectin-dependent adhesion of tumor cells (e.g., Colo201 cells) which express exclusively type 1 chain, i.e., Galβ1→3GlcNAcβ1→3Gal, repeats thereof and substitutions thereof. E-selectin-dependent adhesion of Colo201 cells was inhibited only minimally by mAb CA19-9 (directed to SLe^a) and moderately inhibited by mAb FH7 (directed to disialosyl Le^a and monosialosyl Le^a II). Colo201 adhesion was inhibited most strongly by mAb CA3F4 (directed to monosialosyl Le^a II and Le^a or by a combination of CA19-9 plus CA3F4. Specific reactivities of FH7 with disialosyl Le^a and monosialosyl

Le^a II, and of CA3F4 with monosialosyl Le^a II, were described previously (Nudelman et al., J. Biol. Chem., 261: 5487, 1986).

Further evidence f r the epitope structur s was based on the following observations. Treatment of Colo201 cells with Newcastle Disease Virus (NDV) sialidase, which cleaves NeuAcα2→3Gal (R_1 in Formula I) only slighted inhibit d E-selectin-dependent adhesion, but treatment with Arthrobacter ureafaciens (AU or often denoted as AV in the Figures) or Vibrio cholerae (VC) sialidases, both which cleave NeuAcα2→6 linkage to GlcNAc or Gal (i.e., R_2 in Formula I), completely inhibited such adhesion. Thus, involvement of internally 2→6 sialosylated structures in the adhesion is clear. NDV sialidase in combination with mAb's CA19-9 or CA3F4 strongly inhibited the adhesion. The results described above were obtained in both static and dynamic adhesion systems, described herein.

However, the binding dynamics of selectins is vibrant, as revealed in dynamic flow systems which simulate more closely physiologic conditions, that is, for example, leukocytes or tumor cells can be moving at considerable speed in large and unoccluded small vessels and at a slower speed in occluded vessels and in tissue spaces. Under static conditions cell interactions may be mediated by interaction with a first set of molecules that shar a common characteristic, whereas under non-static conditions, cell interactions may be mediated by interaction with a second set of molecules that share a common characteristic, different from that shared by the first set of molecules. Furthermore, under non-static conditions, the binding requirements may vary depending on the speed at which the cells are moving.

For example, E-sel ctin (ELAM)-mediated adhesion of HL60 cells is dependent on different carbohydrate structures when the cells are reacted in a stationary or slow moving setting or are react d while the cells are in rapidly moving setting. Und r static or low shear conditions ELAM binds preferentially to $\alpha 2 \rightarrow 3$ sialylated and $\alpha 1 \rightarrow 3$ fucosylated structures, such as SLe^x, while under high shear conditions, ELAM preferentially binds to other structures, such as Le^x, Le^y, H and to various hybrid structures, such as Le^x/SLe^x.

Formula II is based on inhibition by various mAB's and sialidases and combinations thereof of E-selectin-dependent adhesion of HL60 tumor cells, which express only type 2 chain, i.e. Galβ1→4GlcNAcβ1→3Gal and repeats thereof, and substitutions thereof. Treatment of HL60 cells with NDV sialidase, which cleaves NeuAcα2→3Gal (R_1 in Formula II), completely abolished reactivity of the cells with anti-SLe^x mAb's, although the cells remained strongly adherent to E-selectin-coated plates and to activated EC's. Complete inhibition of adhesion to E-selectin or EC's required treatment with AU or VC sialidase, which cleav s NeuAcα2→6 linked to GlcNAc or Gal (i.e., R_2 in Formula II, in addition to NeuAcα2→6 as shown in Formula II). The R_5 group is susceptible to cleavage by AU and VC sialidase but not by NDV sialidase.

Further evidence for Formula II was provided by observ d effects of various mAb's on E-selectin-dependent HL60 cell adhesion. The adhesion was inhibited strongly by NDV sialidas in combination with anti-Le^x mAb SH1, or by anti-SLe^x mAb SNH4 in combination with SH1.

The relevance of compounds of structure III was d duced through the use of various mAb's and sialidases on E-selectin-dependent adhesion of tumor cells which express type 2 chain sugars but als with E-selectin-transfected NS-1 cell adhesion to glycoliposomes. For example, NDV sialidase treatment of HL-60 cells, which removes NeuAcα2→6Gal, completely abolish d reactivity of cells with anti-SLe^x mAb although the cells remained adherent to E-selectin plates and activated endothelial cells.

Adhesion was inhibited effectively with a combination of mAb's directed to Le^x and SLe^x.

In contrast to type 1 chain structures whose internally sialosylated structure is known (Nudelman et al., supra) typ 2 chain structures with internally sialic acid residues were hitherto unknown. Data presented in the instant application indicate the natural occurrence of such epitopes.

The structures bindable to ELAM-1 can be synthesized using known techniques. Thus, for example, the carbohydrates can b synthesized chemically using known and commercially availabl reagents or can be synthesized using known and available enzym s to effect the appropriate linkage. For example, known sialosyl transferases and fucosyl transferases can be used to derivatize the basic carbohydrate backbone.

Alternatively, the carbohydrates bindable to ELAM-1 can be isolated using ELAM-1 as an absorbent. For example, purified ELAM-1, cells expressing ELAM-1 or membrane preparations of cells expressing ELAM-1 can be used. Th ELAM-1 can be immobilized t a solid phas , such as an inert bead matrix or the insid wall

of a vessel, to enhance separation. Then suitable carbohydrates bindable to ELAM-1, such as extracts of HL60 or Colo201 cells obtained by known techniques, are exposed t the ELAM-1 affinity matrix. Following a washing procedure to remove unwanted and non-specifically bound components, the ELAM-1 together with carbohydrates bindable thereto are collected. The carbohydrates bound to the ELAM-1 are separated from the ELAM-1, for example, by altering the salt concentration of the holding buffer, and collected. The various carbohydrate species can be discriminat d using known procedures, such as chromatography.

Also, cells known to express predominantly type 1 chain structures or type 2 chain structures are grown and membran preparations are obtained therefrom using known techniques. The glycolipid and glycoprotein fraction of the membrane prep is obtained using known techniques and exposed to an affinity column wherein antibodies directed to carbohydrate epitopes, such as those described herein, are affixed to a matrix, such as agar s beads, to form an affinity matrix. In an affinity chromatography procedure, the bound materials are eluted and separated further by known techniques, such as HLPC and TLC.

When using TLC, the separated molecules in the separati n medium can be exposed to ELAM-1-expressing cells that ar labelled to serve as a tag, for example, the cells can be labelled metabolically with a radioisotope. The ELAM-1-expressing cells will bind to the respective sites of the separation medium where separated ELAM-1 epitopes are found. The TLC matrix can be autoradiographed to locate such sites of cell binding to identify ELAM-1 epitope-bearing molecules. The

respectiv sites of th TLC matrix can be excised and the mol cules extracted.

As noted hereinabove, th carbohydrates of formulae I and II can be derivatized to provid oligosaccharides with mor desirable therapeutic properties. Thus, portions of th structures comprising formula I or II can be substituted, for example, with sulfur-containing sugars or fluorine-containing sugars. The oligosaccharide derivatives can be prepared using the methods disclosed hereinabove but substituting for th naturally occurring components the appropriate reagent comprising an altered substituent, such as 6-trifluoro-fucosyl which is incorporated into either of formula I or II as the fucosyl residues.

The carbohydrates bindable to ELAM-1 can be used as immunogens to obtain antibodies bindable to the carbohydrat s bindable to ELAM-1. Either polyclonal or monoclonal antibodies can be generated, using methods such as those described hereinabove, and in the references cited herein, which ar incorporated by reference. Monoclonal antibodies are preferred.

Because ELAM-1 may serve to mediate intercellular interactions, interruption of binding between ELAM-1 and carbohydrates bindable thereto will be beneficial. Thus, carbohydrates bindable to ELAM-1, ELAM-1, antibody to ELAM-1 or antibody to carbohydrates bindable to ELAM-1, for example, can be used to interrupt binding between ELAM-1 and carbohydrates bindable thereto. The carbohydrates bindable to ELAM-1, ELAM-1, antibody to ELAM-1 or antibody to carbohydrates bindabl to ELAM-1 ar administer d in th rapeutically effectiv amounts and

via routes that are d terminable readily and routinely practicing settled methods f the pharmaceutic arts.

As noted in formulae (I) and (II), the terminal sialic acid is not essential in a carbohydrate bindable to ELAM-1. Key elements held in common are the terminal galactose, glucosamine, α2→6sialic acid and fucose residues. Thus, antibodies capabl of binding to such a structure are effective in inhibiting ELAM-1-mediated interactions. Suitable antibodies are CA3FA and FH7.

Compounds of formula (III), for example, Le^x/SLe^x, wher in relevant epitopes comprised the branched chain structure were identified clearly as comprising high affinity binding sites for ELAM-1 under high shear stress conditions. However, such structures can show less binding ability than simple SLe^x to ELAM-1 at low shear stress conditions or under static conditions. Using that hybrid it is noted that the terminal galactose α1→3 linked fucose to GlcNAc at one branch and an α2→3 linked sialic acid and α1→3 linked fucose at the other branch are critical sites on that hybrid structure. Hence, antibodies bindable to Le^x, such as, SH-1 and FH-2, and to SLe^x, such as FH-6, SNH-4 and SNH-3, are effective cooperatively in inhibiting ELAM-1-mediated adhesion at high shear stress conditions.

Many epitopes recognized by ELAM and GMP-140 are carried by O-linked sugar chains and selectin-dependent cell adhesion can be blocked by inhibitors of O-glycosylation (Kojima et al., Biochem. Biophys. Res. Commun. 182:1288, 1992). Hence, it often is preferable to have compounds of formulae (I), (II) and (III) carried on O-linked carbohydrate chains.

A further m ans of interrupting ELAM-1 mediated interactions is using a c mbination of carbohydrates or antibodies to interfere with ELAM-1 binding to relevant carbohydrates. The carbohydrat s or antibodi s are relat d to ELAM-1 or carbohydrates bindable thereto or in certain circumstances may be carbohydrates or antibodies that are not specifically those carbohydrates believed to bind ELAM-1. For example, a combination of antibodies directed to SLe^x and Le^x is effective in inhibiting ELAM-1 interaction. Suitable SLe^x antibodies ar SNH3 and SNH4; and suitable Le^x antibodies are SH1 and FH2. Th skilled artisan can determine other suitable combinations practicing the methods taught herein using reagents disclos d herein, with particular attention drawn to the working exampl s set forth hereinbelow.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

Example 1
SYNTHESIS OF LACTOSE DERIVATIVES

A. Methyl β-D-lactoside

Heptaacetyllactosylimidate (Zimmermann et al., J. Carbohydr. Chem. 7:435, 1988) was reacted with methanol in dry dichloromethane containing trimethylsilyl trifluor methan sulfonate according to a standard procedure

(Grundler & Schmit, Liebigs. Ann. Chem. 1984:1826, 1984). Purification by silica gel column chromatography (toluene/EtOAc, 1:1 by vol.), followed by de-O-acetylation with 0.01 M sodium methoxid , gave methyl β-D-lactoside in 68% yield fr m th imidate: m.p. 211-212°C (lit. 205°C, Smith & van Cleve, J. Am. Chem. Soc. 77:3159, 1955); $[\alpha]_D$ + 1.3° (c 6.9, H_2O) (lit. + 1°, c 5.0, H_2O), ibid.

B. Phenyl β-D-thiolactoside

Lactose octaacetate (Hudson & Kunz, J. Am. Chem Soc. 47:2052, 1926) was treated with thiophenol and $SnCl_4$ (Nicolaou et al., J. Am. Chem Soc. 110:7910, 1988) in dichloromethane at 0°C to give phenyl heptaacetyl β-D-thiolactoside in 80% yield. The product was deacetylat d with NaOMe in MeOH and neutralized with Amberlyst® 15. Purification of the product on a BioGel® P-2 column using water as an eluent, followed by lyophilization of the sugar-containing fraction, left phenyl β-D-thiolactoside as a white amorphous powder.

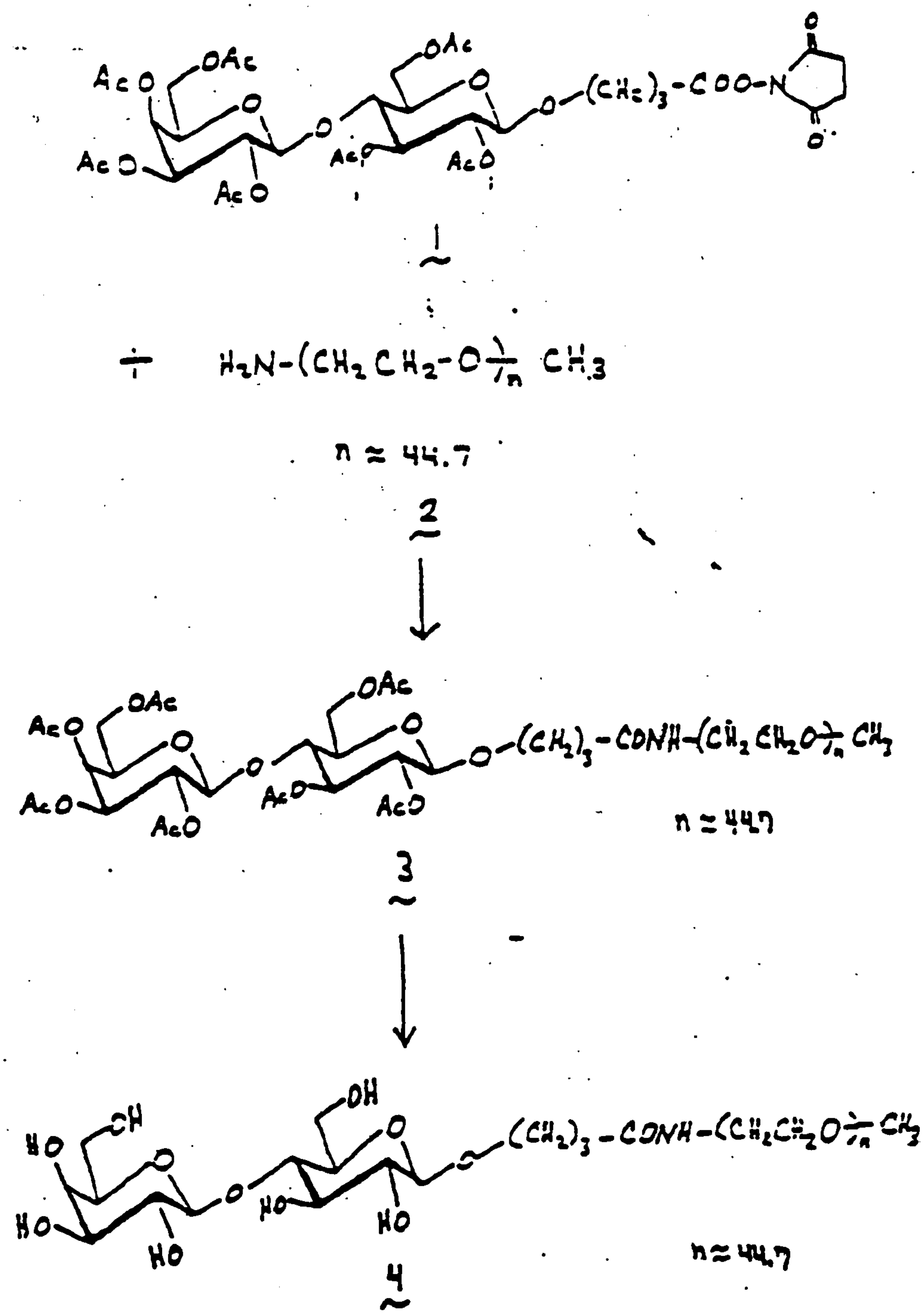
C. Lacto-N-tetrose

The oligosaccharide (Galβ1→3GlcNAcβ1→3Galβ1→4Glc) was prepared from human milk by pretreatment with ethanol and recycling BioGel P-2 column chromatography with water as eluent followed by reversed-phase (C_{18}) high pressure liquid chromatography with water (Dua & Bush, Anal. Bi chem. 133:1,

1983). The ^{1}H-NMR spectrum superimposed that of the authentic sample (BioCarb Chemicals, Lund, Sweden).

D. The poly thylen glycol derivative of β-D-lactoside

The reaction scheme is as set forth below:



The polyethyleneglycol derivative of β-D-lactoside was prepared from readily available 3-succinimidooxycarbonylpropyl O-(2, 3, 4, 6-tetra-O-ac tyl-O-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-O-acetyl-β-D-glucopyranoside 1 and polyethyleneglycol methyl ether (average M.W. 2000; Aldrich Chemical, Milwaukee, WI) having a terminal amino group 2 (Zalipsky et al., Eur. Polym. J. 19:1177, 1983). Treatment of 1 (100 mg, 0.12 mmol) and 2 (163 mg, 0.082 mmol) in dry N,N-dimethylformamide (2 ml) at room temperature for 2 hours gave, after chromatography on LH-20 with acetone as an eluent, the β-D-lactoside heptaacetate 3 in 91% yield: $[\alpha]_D$-5.3° (c 0.5, chloroform). A subsequent saponification of 3 with 0.05 M sodium hydroxide at ro m temperature for one hour, followed by lyophilization, afforded the desired lactoside 4 quantitatively: $[\alpha]_D$-2.4°(c 1.0, chloroform).

Example 2

EFFECT OF LACTOSE AND LACTOSE DERIVATIVES ON METASTATIC POTENTIAL OF B16 MELANOMA CELLS

The highly metastatic BL6 clone of the B16 melanoma cell line was obtained originally from Dr. Jean Starkey (Montana State Univ., Bozeman, MT) and clones were reselected in syngeneic C57Bl mice according to metastatic potential. C57Bl mice were maintained in plastic cages under filtered air atmosphere and provided with water and food pellets ad lib. Cells were cultured in RPMI 1640 supplemented with 2 mM glutamine and 10% fetal calf

serum (FCS), and detached with phosphate buffered saline (PBS) containing 2 mM EDTA. Viability was inferred by a trypan blue exclusion test.

A suspension of BL6 cells (1-3 x 10^6 cells/ml RPMI 1640 medium) was prepared and aliquots were incubated in the presenc or absence of various oligosaccharides at various concentrations, at 37°C for 5-10 minutes. Following incubation, typically, 3 x 10^4 or 2 x 10^4 cells (with or without oligosaccharid pretreatment) per 200 µl were injected via a tail vein into 8-week-old female mice. After 18-21 days, the mice were killed, the lungs were fixed in 10% formaldehyde in PBS (pH 7.4) and tumor cell colonies were counted under a dissecting microscop , thereby providing background values of metastatic melanoma colony number in lung under those conditions. Data on the number and the size of colonies were treated statistically by an analysis of variance (ANOVA) procedure. Colonies with a diameter of 1 mm or greater were considered large-size and those with a diamet r less than 1 mm were considered small-size.

For one experiment, BL6 cells were incubated with various concentrations of lactose, lacto-N-tetrose (Galβ1→3GlcNAcβ1→3Galβ1→4GLc), methyl β-D-lactoside or phenyl β-D-thiolactoside for various durations. In the majority of experiments, a concentration of 0.1 M was used and cells w r incubated at 37°C for 10 minutes, separated from the sugar-containing medium by mild centrifugation at 400 x g for 10 minutes, resuspended in RPMI 1640 and injected (3 x 10^4 cells in 0.2 ml suspension) via a tail vein. For some experim nts, 2 x 10^4 cells were injected and coloni s were counted at 21 days.

Viability and cell growth ability of BL6 cells after incubation in various sugar solutions were tested by trypan blu exclusion test, by plating in RPMI 1640 cultur under normal conditi ns in vitro as well as by subcutaneous inoculation in age-matched C57Bl mice to test tumor growth.

Lactose and lacto-N-tetrose showed 26% and 36% reductions, respectively, of metastatic colonies in lung when BL6 cells wer preincubated with those sugars followed by intravenous injection of cells under identical conditions. Treatment of BL6 cells with 0.1 M, 0.01 M or 0.005 M methyl β-D-lactoside under the sam conditions as above resulted in (respectively) a 43%, 16% and 8% reduction of metastatic lung colony number compared to control. The significant reduction caused by 0.1 M methyl β-D-lactosid was reproduced in three separate experiments and the reduction was found to be consistently between 35% and 45%.

In a second, independent series of experiments, treatment with methyl β-D-lactoside or phenyl β-D-thiolactoside under different conditions also produced a significant reduction f metastatic colonization, i.e., total colony number was reduced to 35% or 50% of control values following preincubation with methyl β-D-lactoside or phenyl β-D-thiolactoside, respectively. Reduction of larger-size colonies was more apparent than that of smaller colonies in all experiments, particularly those with phenyl β-D-thiolactoside (Figure 1). Methyl β-D-lactoside and phenyl β-D-thiolactoside both showed a slight in vitro stimulatory effect on cell number increase and on thymidine incorporation. Thus, the inhibitory effect on tumor deposition is not related to the effect on cell growth in vitro or in vivo.

In a s parate experiment, the effect of m thyl β-D-lactoside on melanoma cell m tastasis was d termined after administration of th oligosaccharide, followed by inoculation with tumor cells. Specifically, a one ml dose of methyl β-D-lactoside (at a concentration of 0.25 M or 0.5 M) was injected intraperitoneally in mice. After 10 minutes, B16 melanoma cells were inject d intravenously. Lung colonies were counted 19 days later. Injection of methyl β-D-lactoside in advance of inoculation with tumor cells resulted in a significant reduction of lung metastatic colony formation (Figure 2).

In a separate experiment, mouse melanoma B16 variants showing different degrees of metastatic potential (BL6/F10/F1/Wa4) showed the same order of expression of GM3 ganglioside, which was previously identified as a melanoma-associated antigen (Hirabayashi et al., J. Biol. Chem. 260:13328, 1985; Nores et al., J. Immunol. 139:3171, 1987). GM3 interacts with LacCer, which is highly expressed on endothelial cells. The order of adhesion of the B16 variants onto LacCer-coated solid phase or onto endothelial cells was also in the same order as metastatic potential (MP). In contrast, integrin-dependent adhesion of the B16 variants was approximately equal for BL6, F10 and F1 (see Figure 4). Those observations suggest that B16 adhesion of LacCer is based on molecular GM3-LacCer interaction. It also has been demonstrated that B16 melanoma adhesion on endothelial cells is inhibited not only by methyl-β-lactoside but also by LacCer liposome, Gg3Cer liposome, and GM3 liposome (see Figure 5).

In addition, the observations on the metastasis-inhibit ry effect of methyl-β-lactoside noted above have been extended to separate methyl-β-lactoside injection, i.e., tumor cells wer injected intravenously, followed by intraperitoneal inj ction of methyl-β-lactoside. In those experiments, injection of 0.25-0.5 M methyl-β-lactoside reduced lung metastatic colony number by 40%-70% (see Figure 6; A = PBS control, B = 0.25 M Me-β-lactoside; C = 0.5 M Me-β-lactoside; D = 0.5 M lactose; E = 0.25 M N-acetyllactosamine; F = 0.5 M Me-β-galactoside; intraperitoneal injection).

Capillary endothelial cells are strongly reactive with antibodies directed to $H/Le^y/Le^b$, such as antibody MIA-15-5. That observation comports with the earlier observations that Ulex Europ. I stains endothelial cells, Holthofer et al., Lab. Invest. 45:391, 1981; 47:60, 1982.

Liposomes comprising H-1 or Le^y were made and exposed to plates to which various glycolipids had been affixed at a rang of concentrations.

As noted in Figure 7, H-bearing liposomes bound to H or Le^y coated onto plates. On the other hand Le^y-bearing liposomes were found to bind only to H-coated plates. H and paragloboside are related, the only difference being the presence of a terminal fucose residue in H.

Hence, cells expressing H, Le^y or Le^b can adhere to endothelial cells expressing H and possibly to Le^y as well. Those types of interactions may be the first step in tumor cell to endothelial cell adhesion.

Example 3

EXPRESSION OF SIALOSYL-DIMERIC LE^X ON HUMAN LUNG ADENOCARCINOMA CELL LINES AND METASTATIC POTENTIAL

KUM-LK-2 is a human non-adenocarcinoma cell lin characterized by producing spontaneous lung metastasis in nude mice. After screening 35 human carcinoma cell lines grown in nude mice, only that cell line produced metastatic deposits in nude mouse lung. KUM-LK-2 was used as the parent cell lin to obtain, by limiting dilution technique, sub-cell lines producing lung metastasis on IV injection.

The procedure for the limiting dilution technique was as follows. KUM-LK-2 was cultured in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% FCS (Hyclone, Logan, UT) at 37°C in a 5% CO_2/95% air atmosphere. Cells were treated briefly with 2 mM EDTA solution and washed twice with RPMI 1640 to make a single cell suspension in RPMI with 10% FCS. Cell viability was > 98% as determined by trypan blue exclusion staining. A cell suspension containing 1 cell per 100 µl was transferred to each well of a 96-well microtiter plate (Corning Glass Works, Corning, NY) and cultured continuously for 24 hours. Each w ll then was examined by phase contrast microscopy.

Three cell lines (HAL-8, HAL-24 and HAL-33) with differ nt metastatic potential ("MP") were selected out of 25 clones obtained by limiting dilution technique on the basis of stable cell morphology. Th 25 clones were sel cted originally from 63

clones showing stable morphology as well as consistent in vitro cell growth.

All f the clones produced spontaneous lung metastasis. However, on I.V. injection, clear differences were observ d among the clones in terms of lung metastatic deposit formation. Two clones with high MP, five with low MP and 18 with no MP were distinguished.

Through repeated selection by I.V. injection of the clones, the most stable sub-cell lines showing consistent MP were established. Those were HAL-8, HAL-33 and HAL-24, showing high, low and no MP, respectively, to nu/nu mouse lung (see Table 1 below). Judging by macroscopic and microscopic examination, none of the three sub-cell lines showed metastasis in other organs or lymph nodes. The sub-cell lines represent stable variants originally present in KUM-LK-2. Based on chromosome analysis, the subclones are independent.

Tabl 1

Metastatic potential f HAL-8, HAL-24 and HAL-33 in nud mice.[a]

Clone	# generations	#lung nodules on day 56[b]
HAL-8	15	15.8 (8-23)
	22	15.0 (10-22)
	46	16.3 (11-25)
HAL-24	15	0
	22	0
	46	0
HAL-33	15	4.3 (3-7)
	22	5.1 (2-8)
	46	5.8 (3-8)

[a] Nude mice were injected (2 x 10^5 cells) via the tail vein at various generation times as indicated. Fifty-six days after injection, mice were killed and metastatic nodules on lung surface were counted under dissecting microscope.

[b]Mean of 6 animals (range in parentheses)

The cell surface expression of various carbohydrate epitopes was analyzed by cytofluorometry using various monoclonal antibodies (mAb's) directed to Le^x (mAb SH1), sialosyl-Le^x (mAb SNH4), sialosyl-dimeric Le^x (mAb FH6), T (mAb HH8), Tn (mAb 1E3) and sialosyl-Tn (mAb TKH2). All antibodies used were culture supernatants from the respective hybridomas, adjusted as 10 µg/ml of immunoglobulin. The structures f sialosyl-Le^x (structure 1), sialosyl-dimeric-Le^x (structure 2), dimeric-Le^x (structure 3), trifucosyl-Le^y (structure 4), Le^b (structure 5), H (structure 6), SA-Le^a I (structur 7),

SA-Tn (structure 8), disial syl-Le^a (structure 9), monosialosyl-Le^a II (structure 10), GM3 (structure 11), S-PG (structur 12), Le^x (structure 13) and Le^a (structure 14) are shown below. R represents a carrier molecule.

<u>Structure 1:</u>

```
NeuAcα2→3Galβ1→4GlcNAcβ1→3[Galβ1→4GlcNAcβ1→3]nGalβ1→4GLcβ1→R
                         3
                         ↑                        (n≥0)
                     Fucα1
```

<u>Structure 2:</u>

```
NeuAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4GLcβ1→R
                          3                 3
                          ↑                 ↑
                      Fucα1             Fucα1
```

<u>Structure 3:</u>

```
Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→R
          3                 3
          ↑                 ↑
      Fucα1             Fucα1
```

<u>Structure 4:</u>

```
Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→R
    2     3                3
    ↑     ↑                ↑        (The α1→2 fucose can be
Fucα1 Fucα1            Fucα1         replaced by H)
```

<u>Structure 5:</u>

```
Fucα1→2Galβ1→3GlcNAcβ1→3Galβ1→R
                4
                ↑
            Fucα1
```

<u>Structure 6:</u>

Fucα1→2Galβ1→3GlcNAcβ1→3Galβ1→R

<u>Structure 7:</u>

```
NeuAcα2→3Galβ1→3GlcNAcβ1→3Galβ1→R
                    4
                    ↑
                 Fucα1
```

<u>Structure 8:</u>

NeuAcα2→6GalNAcα1→O-Ser/Thr

<u>Structure 9:</u>

```
                 NeuAcα2
                    ↓
                    6
NeuAcα2→3Galβ1→3GlcNAcβ1→3Galβ1→R
                    4
                    ↑
                 Fucα1
```

<u>Structure 10:</u>

```
    NeuAcα2
        ↓
        6
Galβ1→3GlcNAcβ1→3Galβ1→R
        4
        ↑
     Fucα1
```

<u>Structure 11:</u>

NeuAcα2→3Galβ1→4Glcβ1→Cer

<u>Structure 12:</u>

NeuAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→R

Structure 13:

```
Galβ1→4GlcNAcβ1→3Galβ1→R
        3
        ↑
     Fucα1
```

Structure 14:

```
Galβ1→3GlcNAcβ1→3Galβ1→R
          4
          ↑
       Fucα1
```

Cells were detached from culture flasks with 0.25% trypsin, 2mM EDTA solution and 1 x 10^5 cells were prepared for each mAb treatment. Cells were incubated with a mAb for 1 hour at 4°C and washed 2 times with RPMI 1640. Goat anti-mouse IgG or IgM-FITC (Boehringer-Mannheim, Indianapolis, IN), diluted 50 times with PBS, then was added and incubated 30 minutes at 4°C. Finally, cells were washed 3 times, resuspended with PBS and analyzed in an EPICS PROFILE flow cytometer (Epics, Hialeah, FL). Th experiments were repeated with three different cell generations.

Patterns of expression of six carbohydrate epitopes (defined by the respective mAb's) on sub-cell lines HAL-8, HAL-24 and HAL-33 showed nearly identical profiles (as did the protein profiles for the three sub-cell lines) except in the case of sialosyl-dimeric-Le^x. In particular, HAL-8, HAL-24 and HAL-33 were found to express highly and equally sialosyl-Le^x and sialosyl-Tn structures. Each of the three lines expressed low quantities of Le^x and Tn, and did not express T. In contrast,

expression of sialosyl-dimeric Le^x was high on HAL-8, moderate on HAL-33 and low on HAL-24.

The release of sialosyl residues was assessed in the following manner. Cells were detached using 2 mM EDTA in PBS, washed and resuspended in 9 volumes of PBS. One ml of cell suspension was incubated 5 minutes at 37°C with 0.2 U/ml of Clostridium perfringens sialidase (type X, Sigma Chemical Co., St. Louis, MO). After incubation, cells were washed three times, resuspended with RPMI 1640 and investigated for MP and expression of sialosyl-dimeric-Le^x. MP of HAL-8 and HAL-33 was inhibited completely by sialidase treatment of cells (see Table 2 below). Expression of sialosyl-dimeric-Le^x appears to play an important role in blood-borne metastasis.

Table 2

Effect of sialidase treatment on metastatic potential of clones HAL-8 and -33.[a]

Treatment	Clone	# lung nodules on day 56[b]
Control (PBS)	HAL-8	16.3 (9-24)
	HAL-33	4.6 (3-7)
Sialidase	HAL-8	0
	HAL-33	0

[a] Nude mice were injected (2×10^5 cells) via the tail vein. Fifty-six days after injection, mice were killed and metastatic nodules on lung surface were counted under dissecting microscope.

[b] Mean of 6 animals (range in parentheses).

EXAMPLE 4

IDENTIFICATION OF CARBOHYDRATE EPITOPES CAPABLE OF BINDING TO THE LECTIN DOMAIN OF GMP-140

Platelets were isolated from "platelet-rich plasma" obtain d from the Oregon Red Cross (Portland, OR). Contaminating red blood cells were removed by centrifugation at 80 x g for 10 min. Platelets were centrifuged at 300 x g for 10 min and suspended in Tyrode's buffer (pH 6.5) containing 22 mM citrate buffer with 0.35% bovine serum albumin (BSA). The platelet suspension (1×10^8/ml) was incubated (pH 7.2, 37°C, 5 min) after addition of thrombin (final concentration 1 U/ml). The mixture then was incubated at 37°C for 10 min without stirring. The thrombin-activated platelets were fixed with an equal volum of 2% formaldehyde in phosphate-buffered saline (PBS), pH 7.2, and washed 2 x with PBS containing 1% BSA. Activated platelets (but not non-activated platelets) showed strong reactivity with 2.5 µg/ml anti-GMP-140 mAb AC1.2 (isotype IgG_1; Beckton-Dickinson, San Jose, CA) when incubated at 37°C for 30 min., followed by reaction with 50 µl of fluorescence-labeled goat anti-mouse Ig (Tago, Burlingame, CA). Flow cytometric profiles of activated vs. non-activated platelets with mab AC1.2 are shown in Figures 8A-8D.

Activated and non-activated platelets were fixed with paraformaldehyde in Ca^{2+}-free PBS, pH 7.2, washed 2 x with Ca^{2+}-containing PBS with 1% BSA, resuspended in CA^{2+}-PBS with 1% BSA and 0.1% azide and the number of platelets adjusted t

$\approx 1\times10^9$/ml. The cell suspension was stored at 4°C and the binding assay performed within 24 hr.

Flu rescent polystyr ne latex beads wer obtained from Molecular Probe, Inc., Eugene, OR. The beads were yellow-gr n fluorescent beads with a sulfate group at the surface, diameter $\approx$ 0.5 μm (actually 0.486 μm). Beads (1×10^9) in 30 μl ETOH w r added to 10 μg of GSL solution in 200 μl C=M, mixed well and dried under an N_2 stream. The residue was resuspended in 200 μl ethanol, sonicated briefly and dried under a N_2 stream. The dried residue was suspended in 2 ml Ca^{2+}-PBS with 3% BSA and 0.1% azide, sonicated for 10 min and allowed to stand at 37°C for 60 min to block the bead surface with BSA. The suspension was centrifuged at 3000 x g for 10 min, the bead pellet was washed 2 x with Ca^{2+}-PBS containing 1% BSA and azide and finally suspended in 500 μl of the same medium and stored at 4°C.

Twenty μl of platelet (non-activated or activat d) suspension, paraformaldehyde-fixed and containing $\approx 2 \times 10^7$ platelets, was mixed with 10 μl of fluorescent GSL-coated beads, containing $\approx 2 \times 10^7$ beads, mixed well and allowed to stand at 37°C for 30 min. The platelet suspension was mixed with 200 μl Ca^{2+}-PBS and analyzed by flow cytometry (EPICS Profile, Coult r Cytometry, Hialeah, FL).

Flow cytometric analyses of platelets alone and beads alon were performed for setting a gating to include most of th signals produced by platelets and excluding signals produced by free beads. The binding index (BI) was calculated as mean fluorescence intensity (MFI) of platelets incubated with fluor scent GSL-coated beads divid d by MFI of platelets

incubated with fluorescent non-GSL-coated (control) beads. BI values for various GSL's are shown in Table 3 and in Figure 9. In Figure 9, the hatched bars represent non-activated platelets and the open bars represent activated platel ts. The ratio of the binding index (BI) of activated/non-activated platelets for SA-Le^x, SA-Le^a, SPG, GM3 and Le^x also is shown in the "Ratio A/NA" column.

Table 3

Binding index of thrombin-activated platelets to GSL-coated, sulfate-containing polystyrene beads

GSL	Activated platelets	Ratio (Activated/ Non-activated)
GM3	1.0 ± 0.1	0.7 ± 0.5
SA-Le^x	4.2 ± 1.0	4.2 ± 0.5
SA-Le^a	8.1 ± 1.0	6.1 ± 0.2
SPG	0.8 ± 0.2	0.7 ± 0.2
Le^x	1.1 ± 0.3	1.0 ± 0.3

[a] Values represent means of four separate experiments.

mAb's affected platelet binding to fluorescent GSL-coated beads. Platelets were incubated with anti-GMP-140 mAb IOP62 (Immunotech, Marseille, France) at 37°C for 30 min and a binding assay was performed using GSL-coated beads, as described hereinabove. Non-specific mouse IgG (10 µg/ml) was used in a control binding assay.

Also, 10 μl of SA-Le^a-coated b ads (2 x 10^7) were incubat d with 20 μl f anti-SA-Le^a mAb CA19-9 (20 μg/ml) (mous IgG_1; Signet Laboratories, Dedham, MA) at room temperature for 60 min and used for th platelet binding assay. Anti-SA-Le^x mAb SNH4 and non-specific mouse IgG were used as controls.

The results are shown in Figure 10, where the abscissa represents percent inhibition and column 1 represents anti-GMP-140 mAb IOP62, column 2 represents anti-SA-Le^a mAb CA19-9 (alternative mAb's are NKH1 and NKH2), column 3 represents anti-SA-Le^x mAb SNH4 and column 4 represents normal mouse IgG.

Activated platelets showed high expression of GMP-140 as evidenced by high reactivity with anti-CD62 mAb (Figures 8A-8D). Activated platelets expressing GMP-140 showed strong binding with fluorescent beads coated with SA-Le^x (Figure 9).

Binding of platelets to beads coated with SA-Le^x was observed but to a much lower degree than with SA-Le^a (Figure 9). No binding was observed to beads coated with other GSL's. Further, the binding of platelets to SA-Le^a coated beads was inhibited by anti-GMP-140 mAb and anti-SA-Le^a mAb, but not by anti-SA-Le^x mAb (Figure 10).

EXAMPLE 5

EFFECT OF VARIOUS MONOCLONAL ANTIBODIES ON ADHESION OF HUMAN COLON CARCINOMA COLO205 CELLS TO INTERLEUKIN-1-ACTIVATED HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS IN A DYNAMIC FLOW SYSTEM

Adhesion was measured using the dynamic flow experimental system shown in Figures 11A to 11D. The number of cells bound during 3 minutes at different shear stresses, for example, from 0.4 to 4.8 dynes/cm^3, were counted from several fields recorded on videotape. The coefficient of viscosity was 1.0 P, the half channel height was 5.7 X 10^{-3} cm and the channel width was 1.3 cm.

Using that system, various human tumors and monoclonal antibodies directed to various tumor-associated carbohydrat antigens were studied. The results of one study, adhesion of human colon carcinoma Colo205 cells to activated human endothelial cells, is shown in Figure 12, where the abscissa represents wall shear stress (dynes/cm^2) and the ordinate represents cell adhesion (x 10^{-2}/field).

In Figure 12, the symbols are as follows: open circles, mixture of irrelevant mouse IgG plus IgM (control); solid triangles, monoclonal antibody CA19-9 directed to monosialosyl-Le^a I; open triangles, monoclonal antibody SNH4 directed to sialosyl-Le^x; solid circles, monoclonal antibody FH7 directed to monosialosyl-Le^a II and disialosyl-Le^a; and solid

squares, mixture f irrel vant mouse IgG plus IgM and non-activat d endothelial cells.

The results show that adhesion of Colo205 cells t activated endoth lial cells was inhibited most strongly by antibody FH7, particularly at high wall shear stress (5-10 dynes/cm^2). In contrast, antibody CA19-9 had no inhibitory effect. The findings suggest that tumor cell adhesion to endothelial cells may proceed via interaction between monosialosyl-Le^a II or disialosyl-Le^a and interleukin-1-activated selectin.

EXAMPLE 6

SELECTIN-DEPENDENT ADHESION OF HL60 CELLS

HUVEC's (Cell Systems, Kirkland, WA) were cultured to confluency in 48-well plates (Costar, Cambridge, MA) and stimulated with 1 U/ml IL-1 for 4 hr. Non-simulated HUVEC's wer used as a control. Expression of E-selectin (ELAM-1) n IL-1-stimulated HUVEC's was confirmed by reactivity with anti-E-selectin mAb 3B7 (IgG_{2a}) (Graber et al. J. Imm. 145:819, 1990). HL60 and Colo201 cells were labeled metabolically by culture in the presence of [^{3}H]-thymidine after pretreatment with glycosylation modifier and added to HUVEC-coated plates. After 15 min incubation, plates were washed with PBS and adherent cell number estimated by conversion from radioactivity count. In another set of experiments, 96-well plates (Falcon, Lincoln, NJ) were coated with 0.1-1 μg/ml of a truncated, recombinant E-s lectin lacking transmembran and cytoplasmic domains (Shimizu

et al. Nature 349:799, 1991) for 18 hr. Plates then were coated with 1% BSA, washed with PBS and coated with metab lically-labeled, glycosylation-modified cells, as described ab ve. After 60 min incubati n, plates were washed with PBS and adherent cell number estimated by conversion from radioactivity count.

Assays of cell adhesion to activated or native platelets coated and fixed on 48-well plates were performed as previously described (Handa et al., Biochemistry 30:11682, 1991). HL60 cells were pretreated with 2mM benzyl-α-GaℓNAc for 72 hr and labeled with [^{3}H]thymidine. After washing with PBS, $1x10^6$ cells were added to each well and plates were incubated for 30 min at room temp. After washing to remove unbound cells, bound cells were detached with trypsin and counted by liquid scintillation counter. Platelets bound on plates were incubated with anti-P-selectin mAb IOP-62 (1:2, 1:6 dilution) (Immunotech, Marseille, France) at room temp for 30 min, followed by addition of HL60 cells, to evaluate dependence of adhesion on P-selectin expression. Non-specific mouse IgG was used as control.

<u>Adhesion assay in a dynamic flow system</u>

A parallel-plate laminar flow chamber connected to an infusion pump (Model 935, Harvard Apparatus, Cambridge, MA) was used to simulate the flow shear stresses present in physiological microvascular environments. The flow chamber consists of a glass plate on which a parallel, transparent plastic surface is attached with a Silastic rubber gasket; there is a 114 μm gap

between the two surfaces and th gap is connected to an inlet and outlet.

A laminar flow with defined rate and wall shear stress is achieved by manipulation of the infusion pump, which is connect d to the inlet of the flow chamber. EC's are grown as a monolayer, or adhesion molecules are coated, on the glass plate, and a laminar flow of a cell suspension is passed through the chamber.

Cell movements are observed under inverted phase-contrast microscope (Diaphot-TMD Nikon) and recorded by time-laps videocassette recorder. Adhesion is observed as rolling follow d by stopping of cells. Number of cells bound during 3 min at different shear stresses from, for example, 0.4 to 4.8 dynes/cm^2 or 0.76 to 15.5 dynes/cm^2 were counted from several fields recorded on videotape. Wall shear stress (T) was calculat d by the equation of Lawrence et al. (Blood 75:227, 1990):

$$T = 3\mu Q/2ba^2$$

where μ= coefficient of viscosity (1.0 cP), Q= volumetric flow rate (cm^3/sec), a= half channel height (for the experiments reported herein, 5.7 x 10^3 cm) and b= channel width (1.3 cm).

<u>HL60 adhesion to E-selectin-coated plates under static conditions</u>

Promyelocytic leukemia cell line HL60 has been shown t express only type 2 chain and sialosylated/fucosylated derivatives as probed by specific mAb's (Symington et al., J. Immunol. 134:2498, 1985) and has been extensively used as a

model of leukocyte adhesion mediated by E-selectin and P-sel ctin (Phillips et al. Scienc 250:1130, 1990; Polley et al. Proc. Natl. Acad. Sci. USA 88:6224, 1991; Handa et al. Biochem. Biophys. Res. Commun. 181:1223, 1991; Kojima et al. Biochem. Biophys. Res. Commun. 182:1288, 1992).

When HL60 cells were treated with Newcastle Disease Virus (NDV) or Vibrio cholerae (VC) sialidase, reactivity of cells with mAb's SNH3 and SNH4 was abolished (Figure 13). E-selectin-dependent HL60 adhesion was reduced by only about 20-50% after treatment with NDV sialidase, whereas adhesion of the same cells treated with Vibrio or Arthrobacter ureafaciens (AU) sialidase was reduced to about 5-10% of control values or essentially abolished. (NDV, VC and AU sialidase were effective equally in eliminating SLe^x expression on HL60 cells.)

NDV sialidase eliminates only the $\alpha 2 \rightarrow 3$ sialosyl residue linked to the terminal Gal whereas both Vibrio and Arthroacter sialidase completely eliminate terminal and internal sialic acid residues, notably, $\alpha 2 \rightarrow 6$ linked sialic acid residues. Th findings indicate that SLe^x and SLe^a are not the sole epitopes of E-selectin and P-selectin.

Effects of various mAb's on E-selectin-dependent HL60 adhesion were tested. Anti-SLe^x mAb's SNH3 and SNH4 produc d strong HL60 cell aggregation, even under carefully-controll d conditions. Therefore, the degree of inhibition of HL60 adhesion by SNH3 or SNH4 varied considerably since aggregated cells tend to detach from E-selectin-coated plates.

In general, th degree of inhibition by th se mAb's was minimal compared to the degre of inhibition previously describ d

by Phillips et al. (supra). Anti-Le^x mAb's SH1 (IgG_3) (Singhal et al. Cancer Res. 50:1375, 1990) and FH2 (IgM) (Fukushi et al. J. Biol. Chem. 259:4681, 1984) produced a consist ntly high r degr e of inhibition than mAb's SNH3 and SNH4.

A mixture of SNH3 or SNH4 with SH1 or with FH2 produc d stronger inhibition than any of the mAb's alone. Strongest inhibition was produced with a mixture of SNH4 and SH1 (Figure 14).

If SLe^x is the sole epitope of HL60 cells for E-selectin and P-selectin, anti-SLe^x mAb's (e.g., SNH3 and SNH4) should inhibit completely selectin-dependent adhesion. Treatment with NDV sialidase, which abolished reactivity of HL60 cells with SNH3 and SNH4, also should inhibit E-selectin-dependent cell adhesion. However, treatment of HL60 cells with NDV sialidase followed by SNH3 or SNH4 did not further reduce adhesion. Treatment with NDV sialidase followed by anti-Le^x mAb's SH1 or FH2 strongly inhibited E-selectin-dependent HL60 adhesion.

<u>HL60 adhesion to activated HUVEC's in a static system</u>

The same trends observed for HL60 adhesion to E-selectin-coated plates were observed for HL60 adhesion t activated HUVEC's grown in plates. Adhesion to HUVEC's was affected minimally by anti-SLe^x mAb's SNH2 or SNH4, in contrast to the previous report by Phillips et al. (supra). Different preparations of the mAb's varied widely in the effect on HL60-HUVEC adhesion, and some mAb's caused strong aggregation of HL60 cells. Anti-Le^x mAb's SH1 and FH2 showed consistently

stronger (compared to SNH3 r SNH4) inhibition of HL60-HUVEC adhesion, as did a combination of SNH4 plus SH1 or FH2. NDV sialidase did not reduce significantly HL60-HUVEC adh sion, but Vibrio sialidase almost abolished reactivity completely.

<u>Coating of adhesion molecules or EC's on glass plates in the dynamic flow system</u>

For lectins, fibronectin (FN), laminin (LN), truncat d E-selectin and GSL's used, 10-50 μl of a solution having a concentration of 20-200 μg/ml was placed on a marked area (0.5 cm diameter) on a glass plate (38 x 75 mm; Corning Glassworks, Corning, NY) and dried in a refrigerator at 4°C. Dried plates were immersed in PBS at 37°C for 1 hr and washed extensively with several changes of PBS. For GSL coating, GSL-liposomes were prepared from 200 μg GSL, 200 μg cholesterol and 400 μg phosphatidylcholine in 1 ml PBS. Ten μl of GSL-liposome soluti n was placed on a glass plate, dried at 4°C and the plates wer washed with PBS, as described above.

The quantity of adsorbed molecules was determined using ^{125}I labeling for lectins, FN or LN, or [^{3}H]cholesterol labeling for GSL-liposomes. Under those conditions, almost the entire quantity of protein, regardless of whether FN, LN or lectin, was adsorbed on the glass plate. For example, when 100 μg/ml FN was applied, 12.5 $\pm$ 1.8 ng/mm^2 was adsorbed. Likewise, almost all GLS-liposome dried on the glass plate was adsorbed; e.g., when 200 μg/ml GLS-liposom was appli d, 31.3 $\pm$ 5.2 ng GSL/mm^2 was adsorb d.

EC's w r coated by placing 100 μl of a suspension containing 2 x 10^5 mous or human EC's on glass plates and culturing in a CO_2 incubator at 37°C until confluency was achiev d.

Plates coated with adhesion molecules or EC's were affix d in a flow chamber, and a suspension of B16 melanoma cells was passed through the chamber as described hereinabove. B16 cells were harvested from culture using 0.02% EDTA in PBS, and suspended in PBS at a concentration of 1 x 10^5/ml.

<u>HL60 adhesion to activated HUVEC's in a dynamic flow system</u>

The effects of various mAb's and sialidases on HL60-HUVEC adhesion were tested also in a dynamic flow system. Generally, the effects of the mAb's were similar to those observed with a static system. mAb SNH4 had no inhibitory effect at all under various shear stresses. mAb's SH1 and FH2 showed moderate inhibition. Again, strongest inhibition was obtained with a combination of SNH4 plus SH1 or FH2. Adhesion was reduced moderately by NDV sialidase and almost completely by Vibrio or Arthrobacter sialidase. See Figure 15.

The results set forth hereinabove using HL60 cells suggest that the presence of sialic acid in the carbohydrate epitope is important in providing binding specificity to E-selectin. However, the sialic residue is not required to be $\alpha 2\rightarrow 3$ linked at the terminal Gal; the sialic acid residue alternatively could b present at an internal location, e.g., link d t internal Gal or

GlcNAc. Clearly, though, $\alpha1\rightarrow3$ fucosylation at GlcNAc is essential.

Under dynamic conditions, NDV sialidase had an inhibitory effect only at low shear stress whereas VC or AU sialidase significantly reduced adhesion even at high shear stress. Anti-Le^x IgG mAb SH1 strongly inhibited adhesion even at high shear stress, whereas the effect of anti-SLe^x IgG_3 mAb SNH4 was minimal. Strongest inhibition was produced by a combination of NDV sialidase plus anti-Le^x mAb SH1. A mixture of anti-Le^x plus anti-SLe^x mAb's produced stronger inhibitory effect than either mAb alone.

As depicted in Figure 19, at low shear str ss (<4 dynes/cm^2), adhesion was inhibited significantly by NDV sialidase or by mAb SNH4, whereas those reagents had no eff ct at high shear stress (8-16 dynes/cm^2). In contrast, VC sialidas completely abolished adhesion at high shear stress. mAb SH1 inhibited adhesion more strongly at high than at low shear stress, but the difference was relatively small.

Le^x alone clearly is not sufficient as the E-selectin epitope. Rather, $\alpha2\rightarrow3$ plus $\alpha2\rightarrow6$ sialylated structures are necessary. Le^x-liposomes and Le^a-liposomes bind to ELAM-coat d plates, and binding by SLe^x is stronger than by Le^x or other glycolipids. However, those epitopes must be present at the cell surface in the form of multiply O-glycosylated mucin-type glycoproteins. Le^x as well as SLe^x may be $\alpha2\rightarrow6$ sialylated at the internal Gal or GlcNAc within the same CHO chain, or $\alpha2\rightarrow6$ sialylation may be present at an adjacent branched structure. "6-C ganglioside," which is an $\alpha2\rightarrow6$ sialylat d typ 2 chain

structure with internal α1→3 fucosylation (Hakomori t al., Biochem. Biophys. Res. Commun. 113:791, 1983), failed to bind to E-selectin. Thus, such a structure can be excluded as a possible ELAM epitop .

EXAMPLE 7

COLO201 CELL ADHESION: EFFECTS OF VARIOUS SIALIDASES AND mAb's

<u>Colo201 adhesion to E-selectin-coated plates under static conditions</u>

In contrast to HL60 cells (which express predominantly type 2 chain structure), Colo201 cells express mainly type 1 chain, and E-selectin-dependent Colo201 adhesion is through type 1 chain epitopes. Colo201 cells were treated with various mAb's following exposure to various sialidases and were ass ssed for residual binding. Colo201 reactivity with mAb CA19-9 (directed to SLe^aI) was inhibited almost completely by Vibrio sialidase, and to a lesser extent by Arthrobacter and NDV sialidases.

In contrast, Colo201 reactivity with mAb FH7 (directed to di-SLe^a and SLe^a II) was reduced by Arthrobacter sialidase but minimally affected by Vibrio or NDV sialidases. Col 201 reactivity with mAb CA3F4 (Nudelman et al. J. Biol. Chem. 261:5487, 1986) was enhanced by sialidase treatment. mAb CA19-9

inhibited Colo201 adhesion slightly and was influenced only minimally by Vibrio sialidase (Figure 16).

It is possible that the SLE[a] epitope present at the surface of C lo201 cells is organiz d in such as way that it is (i) not susceptible to CA19-9 for E-selectin-dependent adhesion and (ii) not sensitive to sialidase treatment. The inhibitory effects of mAb FH7 (Nudelman et al. supra) and, more strikingly, mAb CA3F4 on Colo201 adhesion to E-selectin-coated plates were enhanced by pretreatment of cells with Vibrio sialidase. Arthrobacter sialidase reduced but did not abolish Colo201 adhesion. See Figure 17.

<u>Colo201 adhesion to E-selectin-coated plates in a dynamic flow system</u>

In the dynamic flow system, NDV sialidase had no effect n Colo201 adhesion, particularly at high shear stresses. Anti-SLe[a] I mAb CA19-9 had no effect, whereas anti-SLe[a] II mAb FH7 had a moderate inhibitory effect, in agreement with results from the static system.

At both low and high shear stresses, the strongest inhibition of adhesion was observed for mAb CA3F4, which is directed to Le[a] with an $\alpha 2 \rightarrow 6$ sialosyl substitution at the penultimate GlcNAc. Vibrio sialidase, which efficiently cleaves terminal $\alpha 2 \rightarrow 3$ sialosyl linkages but is less effective at removing internal sialic acid residues, reduced adhesion to some extent at high shear stress, but less so at low shear stress.

Similarly, mAb CA3F4 inhibit d adhesion strongly at high shear stress but much less at low shear stress. A combination f Vibrio sialidas plus mAb CA3F4 produced strong inhibition at both high and low shear stress (Figure 18).

A similar trend was observed for NDV sialidase, which specifically cleaves terminal $\alpha 2 \rightarrow 3$ sialosyl linkages. Colo201 adhesion, at either high or low shear stress, was not affected by NDV sialidase alone, nor by NDV sialidase followed by mAb CA19-9. In contrast, adhesion was inhibited strongly, at both high and low shear stress, by NDV sialidase followed by mAB FH7 or CA3F4.

Rolling velocity (μm/sec) of Colo201 cells along E-selectin-coated plates was evaluated after treatment with various sialidases and mAb's. At three different shear stresses, velocity was unaffected by NDV sialidases and mAb CA19-9; i. ., cells once stopped (0 μm/sec) did not start rolling again. However, Vibrio or Arthrobacter sialidase, or mAb CA3F4, caus d once-stopped cells to start rolling again; the resulting velocity depending on shear stress. The greatest velocity resulted from treatment with a combination of CA3F4 plus Vibrio sialidase.

<u>Colo201 adhesion to activated HUVEC's in a static system</u>

As noted for Colo201 adhesion to E-selectin-coated plates, mAb's CA19-9 and FH7 had negligible effect, but mAb CA3F4 strongly inhibited adhesion. Treatment of cells with NDV sialidase, which cleaves terminal $\alpha 2 \rightarrow 3$ sialosyl linkage, either bri fly or for 24 hr, did not reduc adhesion significantly.

Treatment for 24 hr with Vibrio sialidase completely abolished adhesion. Brief treatment with Vibrio and Arthrobacter sialidase still reduced adhesion significantly.

Colo201 adhesion to activated HUVEC's in a dynamic flow system

Inhibition of adhesion was strongest for mAb CA3F4 and non-existent for mAb CA19-9. mAb INH1 or ST421, directed to unsubstituted type 1 chain (Stroud et al. J. Biol. Chem. 266:8439, 1991), also caused significant inhibition. Adhesion was unaffected by NDV sialidase but strongly inhibited by Vibrio sialidase.

The observed effects of sialidases and mAb's on Colo201 adhesion to E-selectin-coated plates and to HUVEC's suggest that the type 1 chain epitope recognized by E-selectin is internally sialosylated and fucosylated.

EXAMPLE 8

Truncated, recombinant ELAM-1 lacking the transmembrane and cytoplasmic domains is used to coat beads, for example, capable of packing into a standard chromatography columns. The ELAM-1 at a concentration of 0.1-1 μg/ml is mixed with the beads and the mixture is incubated to allow binding of ELAM-1 to the bead matrix. A suitable incubation period is 12-24 hours at 4°C - room temperature. The beads are washed to remove unbound ELAM-1, optionally can be blocked with an inert carrier, such as BSA, and washed again.

The ELAM-1 coated beads can be used in a batch process or packed into a suitably-sized column.

Cells known to carry carb hydrates bindabl to ELAM-1, such as HL60 and Colo201, are obtained. The cells ar lys d to obtain a membrane fraction using known methods, such as repeated freeze-thaw cycles. The membrane fraction is obtained, for example, by centrifugation.

If the cell source is known to express only or predominantly carbohydrates bindable to ELAM-1, the membrane prep may b a suitable source without further purification.

The membrane prep is treated using known methods to obtain a membrane component preparation, and in particular, a fraction that contains cell surface carbohydrate. The carbohydrate-rich fraction is mixed with or passed over the ELAM-1 affinity matrix, depending on the format, the exposed matrix is washed and the carbohydrates bound to the matrix are eluted, for example, by exposing the matrix to a high salt buffer.

The resultant preparation comprises carbohydrate bindable to ELAM-1 and the various species are separable using known techniques, such as TLC or HPLC.

EXAMPLE 9

Carbohydrates bindable to ELAM-1, either prepared chemically using known reagents and methods, see, for example, Example 1 hereinabove, prepar d enzymatically or obtained from suitabl c lls, s e, for example, Exampl 8 hereinabove, or whole cells

known to express carbohydrat bindabl to ELAM-1, serve as immunogen in suitable hosts to generate antibody ther to. Eith r polyclonal or monoclonal antibody can be obtain d and the selection of a suitable host is premis d on known methods and preferences. The carbohydrates, cells, cell lysates or membran preps are administered to the host, either with or without adjuvant, in a schedule that will generate an immune response.

In the case of polyclonal antisera, the blood is collected, serum separated and tested.

In the case of monoclonal antibodies, the spleens of th host animals are removed and cells therefrom are fused with a suitable myeloma cell using known techniques.

Specificity of the antibodies can be tracked using an ELISA comprising, for example, purified recombinant ELAM-1 and mAb 3B7 with the appropriate labeled reagents and reporter molecules.

Antibody directed to carbohydrates of formulae (I), (II) and (III) can be obtained by using specific carbohydrate species as antigen and in the screening ELISA.

Alternatively, the antisera can be made "monospecific" by absorption with cells carrying only SLe^x and/or SLe^a or with a solid matrix to which SLe^x and/or SLe^a is bound. The resultant residual activity directed to carbohydrates bindable to ELAM-1 can be attributed in part to antibodies directed to carbohydrates of formula (I), (II) or (III).

EXAMPLE 10

NS-1 cells were obtained from th ATCC (Rockvill , MD) and maintain d in RPMI 1640:Dulbecco's MEM (1:1) supplement d with 10% HI FCS. Fifty μg of a plasmid comprising cDNA of E-selectin in vector pCDM8 (R & D Systems, Minneapolis, MN) and 5 μg f pSV2-neo (ATCC) were co-transfected into NS-1 cells (1×10^7) by electroporation. After 48 hours in culture, the cells were transferred to medium containing 650 μg/ml G418 (Gibco, Grand Island, NY).

After 15-20 days, resulting colonies were screened for E-selectin expression by staining with mAb (obtained from W. Newman, Maryland Research Laboratories, Otsuka Pharmaceutical Co., Rockville, MD). The variant expressing the highest level of E-selectin was isolated by panning with mAb followed by limiting dilution to achieve clonality.

E-selectin-dependent adhesion using transfected NS-1 cells onto SLe^a, SLe^x, Le^y, Le^x, H-2, sialylparagloboside (SPG), disialosyl I (Structure 6 of Figure 20) and dimeric SLe^x were compared at various shear stress conditions. The number of cells adhered per mm^2 is expressed relative to adhesion onto SLe^x-coated plates which is regarded as 100%.

Whereas adhesion to Le^y and Le^x slightly increased at high shear stress, the absolute numbers of cells which adhered was much lower at both low and high shear stress conditions relative to that observed with SLe^x and SLe^a. Adhesion with structure 1 of Figure 20 was enhanced at middle and high shear stress conditions.

The high binding capacity of structure 1 f Figure 20 was revealed further using 1 w concentrations of glycolipids in liposomes (glycoliposomes). Transfected NS-1 adhesion on SLe^x lip somes was enhanc d when Le^x or Ley was add d and present d as a mixed glycoliposome. The results of the experiments are presented in Figures 21-24.

From the foregoing, it will be evident that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be mad without deviating from the spirit and scope of the invention.

All references cited herein are incorporated by reference.

WHAT IS CLAIMED IS:

1. A carbohydrate or substituted derivative thereof having the formula:

```
               NeuAcα2              R2          R2          R2
                   ↓                |           |           |
                   6                |           |           |
Galβ1→4GlcNAcβ1[→3Galβ1→4GlcNAcβ1→]n3Galβ1,
 |                 3                            |
 R5                ↑                            R4
                Fucα1
```

wherein R_2 is H or an α2→6 linked sialic acid, R_4 is H or an α1→3 linked fucose, R_5 is H, an α2→3 linked sialic acid, an α2→3 linked NeuAcα2→8NeuAc disaccharide or an α2→3 linked R_6-sialic acid carbohydrate, wherein R_6 is one or more sugars, and n is equal to or greater than 0.

2. A carbohydrate or substituted derivative thereof having the formula:

```
               NeuAcα2              R2          R2
                   ↓                |           |
                   6                |           |
Galβ1→3GlcNAcβ1[→3Galβ1→3GlcNAcβ1→]n3Galβ1,
 |                 4                            |
 R1                ↑                            R3
                Fucα1
```

wherein R_1 is H or an α2→3 linked sialic acid, R_2 is H or an α2→6 linked sialic acid, R_3 is H or an α1→4 linked fucose, and n is equal to or greater than 0.

3. A carbohydrate or substituted derivative thereof having the formula:

$$\begin{array}{l} R_{10}1\rightarrow6 \\ \qquad\qquad R_8\text{—}R_9 \\ R_{11}1\rightarrow3, \end{array}$$

wherein each of R_{10} and R_{11} comprises galactose, Galβ1→4GlcNAc or Galβ1→3GlcNAc; R_8 comprises galactose or GalNAc; and R_9 comprises lactosyl ceramide or an oxygen group of a lipid or a protein which bonds carbohydrate.

4. The carbohydrate of claim 3, wherein R_{10} comprises the Le^x epitope and R_{11} comprises the SLe^x epitope.

5. The carbohydrate of claim 4, wherein R_8 is Galβ1→4GlcNAc.

6. The carbohydrate of claim 5, wherein R_9 is an oxygen group of a lipid or a protein which bonds carbohydrate.

7. The carbohydrate of claim 3, wherein R_{10} comprises the Le^a epitope and R_{11} comprises the SLe^a epitope.

8. The carbohydrate or substituted derivative of claim 3 which is:

```
           Fucα1
               ↓
               3
Galβ1→4GlcNAcβ1→6
                      Galβ1→3GlcNAcβ1→R  .
Galβ1→4GlcNAcβ1→3
 3             3
 ↑             ↑
SA2        Fucα1
```

9. An antibody which binds specifically to a carbohydrate or substituted derivativ thereof of any one of claims 1-8.

10. The antibody of claim 9, wherein said carbohydrate is the carbohydrate of claim 4.

11. The antibody of claim 9, wherein said carbohydrate is the carbohydrate of claim 7.

12. The antibody of claim 8, wherein said carbohydrate is the carbohydrate of claim 8.

13. A composition comprising at least two carbohydrates that are involved in tumor cell or leukocyte adhesion to endothelial cells.

14. The composition of claim 13, wherein one of said at least two carbohydrates is SLe^x.

15. The composition of claim 14, which further comprises Le^x.

16. The composition of claim 14, which further comprises Le^y.

17. The composition of claim 13, wherein said at least two carbohydrates comprise Le^a and SLe^a.

18. The composition of claim 13, which further comprises a liposome.

19. A composition comprising at least two antibodies, wherein each of said two antibodies specifically binds to one of said at least two carbohydrates comprising a composition of any one of claims 13-17.

20. The composition of claim 19, wherein an antibody specifically binds to SLe^x.

21. The composition of claim 20, which further comprises an antibody which specifically binds to Le^x or Le^y.

22. The composition of claim 19, wherein an antibody specifically binds to SLe^a.

23. The composition of claim 22, which further comprises an antibody which specifically binds to Le^a or Le^b.

24. A method for interrupting intercellular interactions mediated by ELAM-1 with cells expressing type 1 chain comprising at the terminus:

$$\begin{array}{c} \mathrm{NeuAc}\alpha 2 \\ \downarrow \\ 6 \\ \mathrm{Gal}\beta 1\rightarrow 3\mathrm{GlcNAc} \\ 4 \\ \uparrow \\ \mathrm{Fuc}\alpha 1, \end{array}$$

comprising exposing said cells t an antibody which binds specifically to Le^a.

25. The method of claim 24 wherein said antibody is CA3FA or FH7.

26. A method for interrupting ELAM-1-mediated intercellular interactions between cells comprising exposing said cells to at least one antibody which binds specifically to a carbohydrat bindable to ELAM-1.

27. The method of claim 26 wherein said carbohydrate bindable to ELAM-1 is SLe^x, hybrid Le^x/SLe^x or hybrid Le^a/SLe^a.

28. The method of claim 27 wherein said carbohydrate has the structure:

```
               Fucα1
                   ↓
                   3
Galβ1→4GlcNAcβ1→6
                   Galβ1→3GlcNAcβ1→R   .
Galβ1→4GlcNAcβ1→3
 3                 3
 ↑                 ↑
SA2           Fucα1
```

29. The method of claim 26, which comprises an antibody which binds specifically to SLe^x.

30. The method of claim 29, which further comprises an antibody which binds specifically to Le^x.

31. The method of claim 26, which comprises an antibody which binds specifically to SLe^a.

32. The m thod of claim 31, which further comprises an antibody which binds specifically to Le^a.

33. The method of claim 26 which further comprises an antibody which binds specifically to a carbohydrate not bindable to ELAM-1.

34. The method of claim 33 wherein said carbohydrate not bindable to ELAM-1 is Le^x, Le^y, Le^a or Le^b.

35. The method of claim 30 wherein said antibody which binds specifically to SLe^x is SNH3 or SNH4 and said antibody which binds specifically to Le^x is SH1 or FH2.

36. A method for interrupting ELAM-1-mediated intercellular interactions between cells comprising exposing said cells to at least one member of the group consisting of ELAM-1, carbohydrate bindable to ELAM-1, sialidase, antibody which binds specifically to ELAM-1 and antibody which binds specifically to carbohydrate bindable to ELAM-1.

37. The method of claim 36, wherein said carbohydrate bindable to ELAM comprises an $\alpha 2\rightarrow 3$ linked sialic acid.

38. The method of claim 36, wherein said carbohydrate bindable to ELAM is Le^x.

39. The m thod of claim 36, wherein said carbohydrate bindable to ELAM comprises an α2→6 linked sialic acid.

40. The method of claim 36, wherein said antibody which binds specifically to carbohydrate bindable to ELAM is an antibody which binds specifically to carbohydrate bindable to ELAM comprising an α2→3 linked sialic acid.

41. The method of claim 36, wherein said antibody which binds specifically to carbohydrate bindable to ELAM is an antibody which binds specifically to carbohydrate bindable t ELAM comprising an α2→6 linked sialic acid.

42. The method of claim 36, wherein said antibody which binds specifically to carbohydrate bindable to ELAM is an antibody which binds specifically to Le^x.

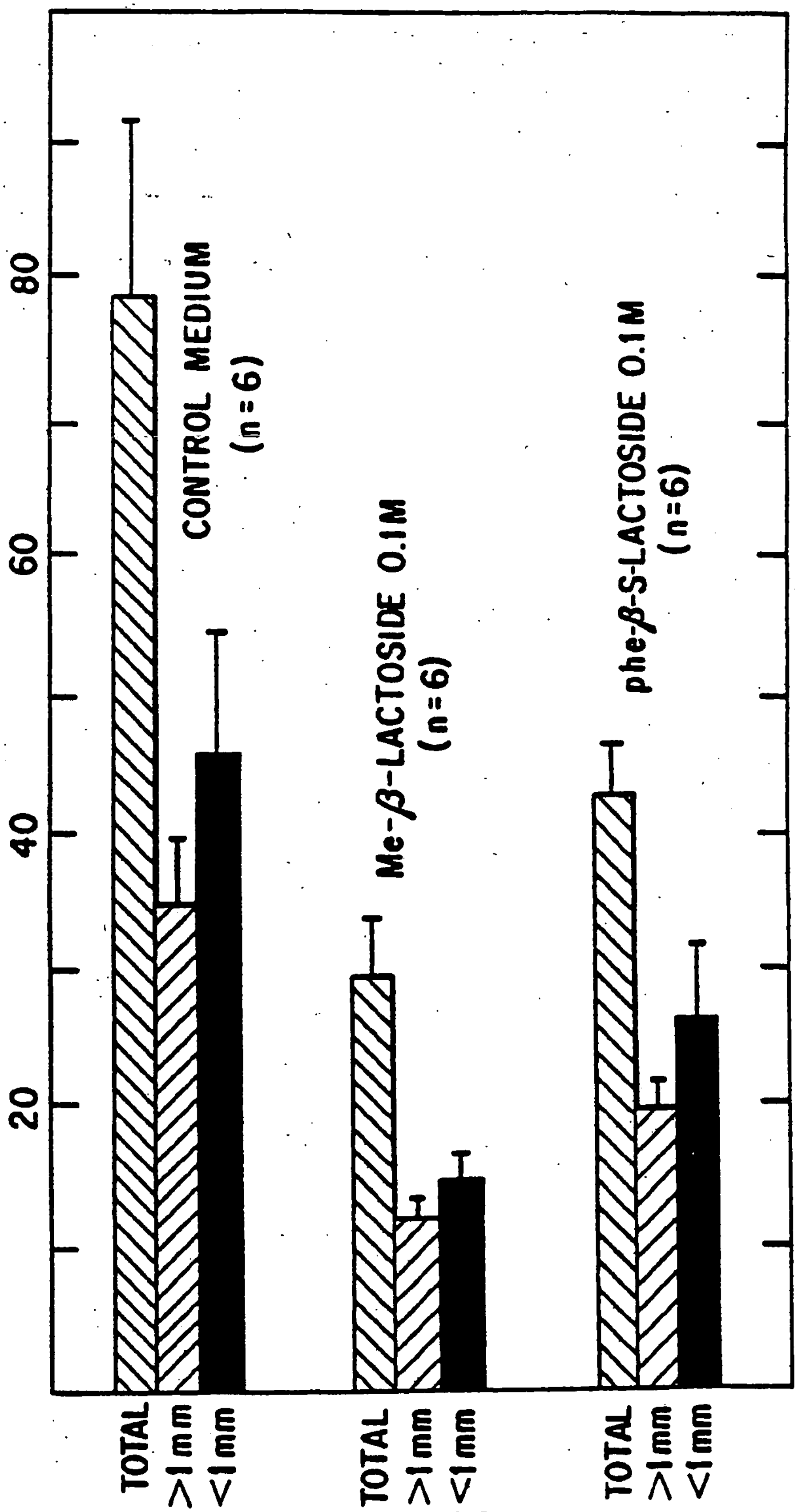

CONTROL MEDIUM
(n=6)
Me-β-LACTOSIDE 0.1 M
(n=6)
phe-β-S-LACTOSIDE 0.1 M
(n=6)
TOTAL
>1 mm
<1 mm
20
40
60
80

FIG. 1

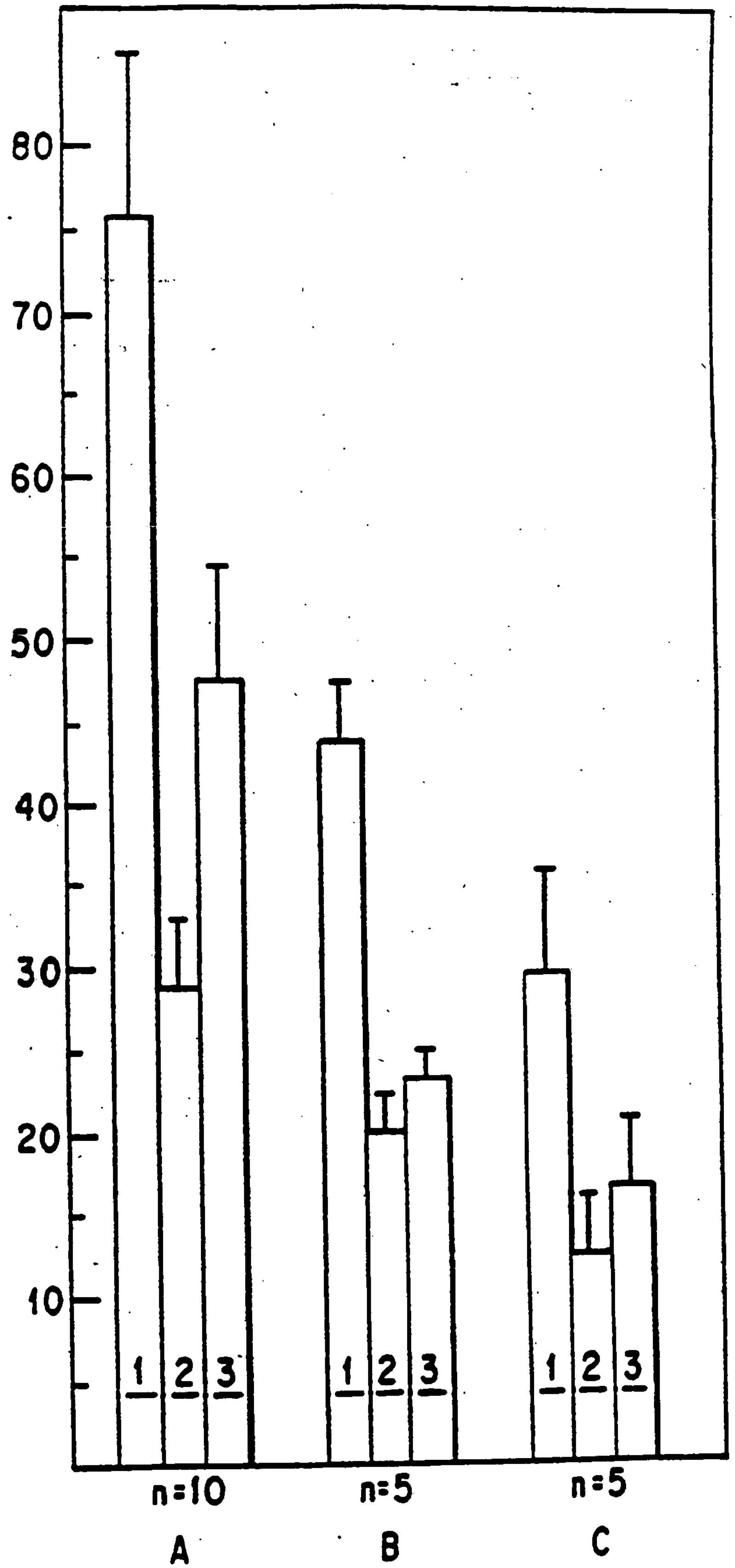


FIG. 2

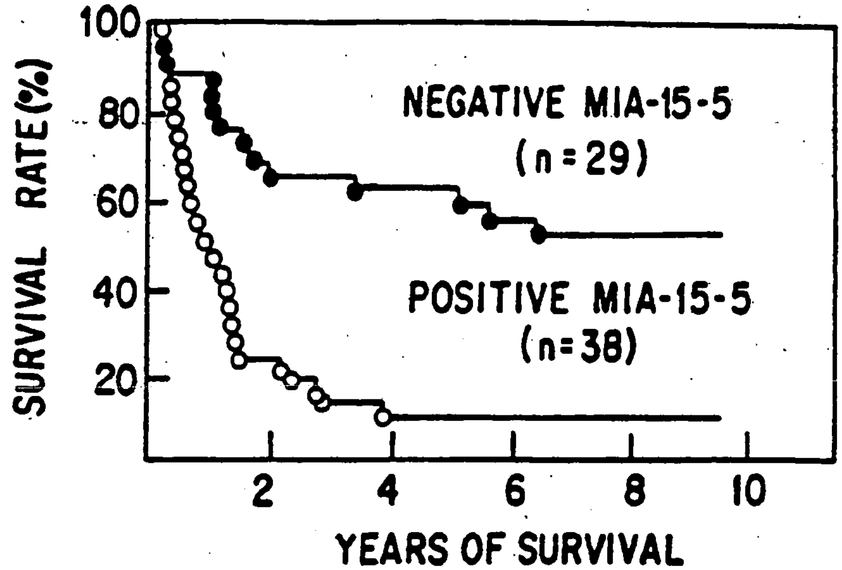

100
80
60
40
20
SURVIVAL RATE (%)
NEGATIVE MIA-15-5
(n=29)
POSITIVE MIA-15-5
(n=38)
2
4
6
8
10
YEARS OF SURVIVAL

FIG. 3A

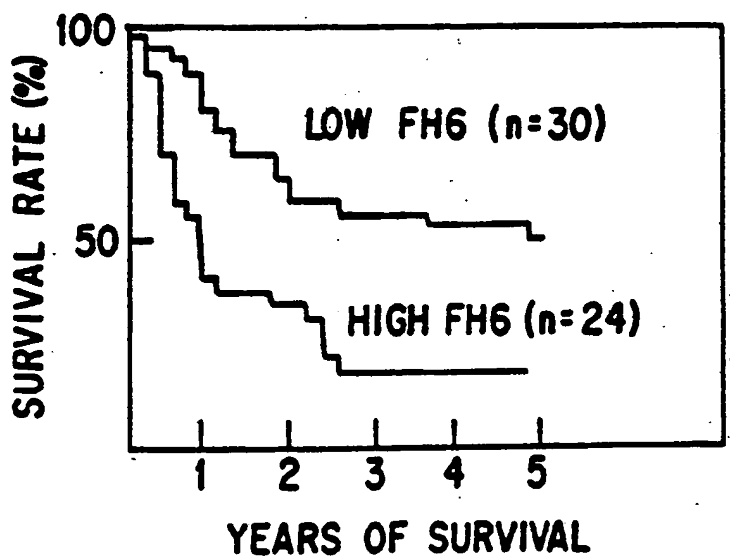

100
50
SURVIVAL RATE (%)
LOW FH6 (n=30)
HIGH FH6 (n=24)
1
2
3
4
5
YEARS OF SURVIVAL

FIG. 3B

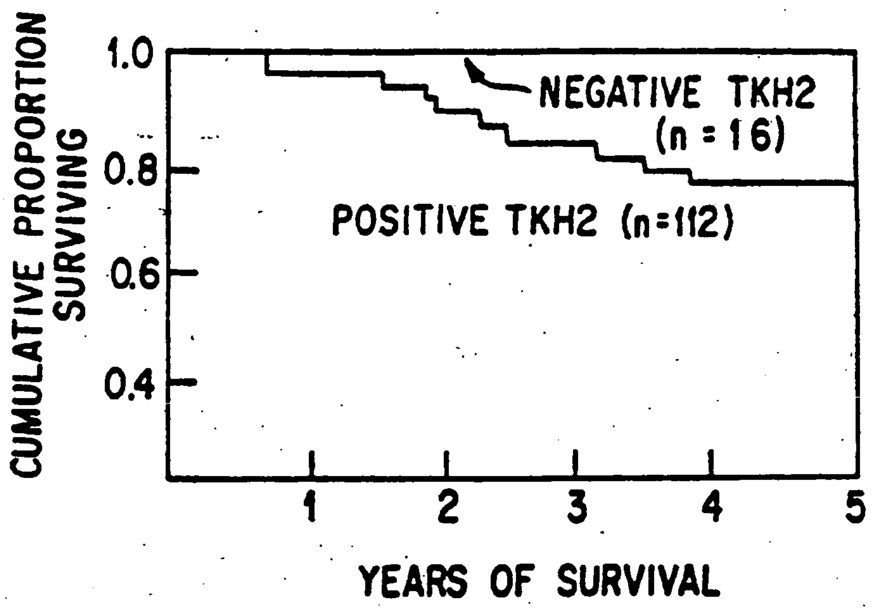

CUMULATIVE PROPORTION SURVIVING
1.0
0.8
0.6
0.4
NEGATIVE TKH2 (n = 16)
POSITIVE TKH2 (n=112)
1
2
3
4
5
YEARS OF SURVIVAL

FIG.3C

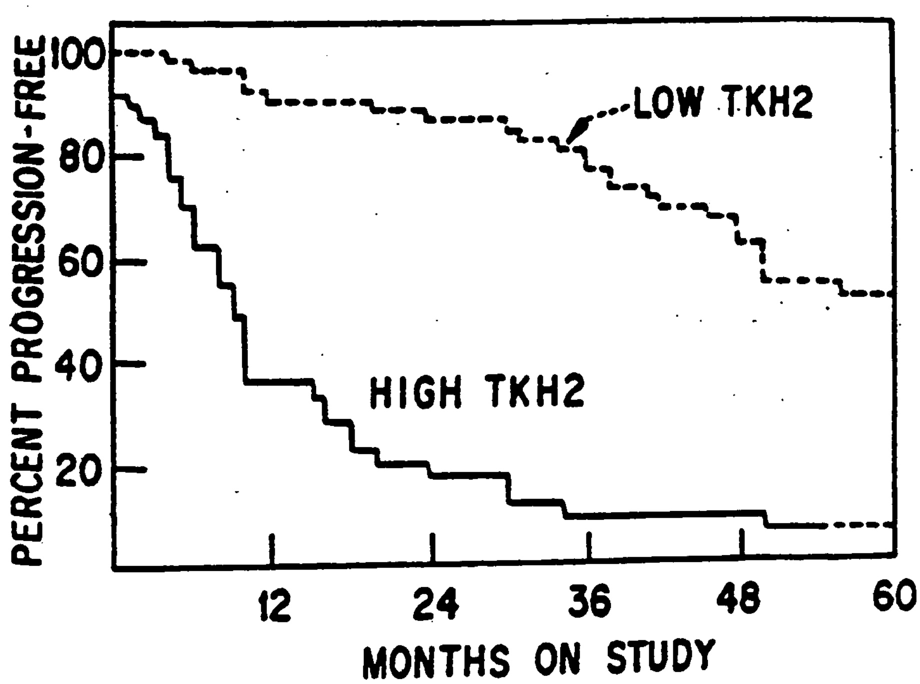

PERCENT PROGRESSION-FREE
100
80
60
40
20
LOW TKH2
HIGH TKH2
12
24
36
48
60
MONTHS ON STUDY

FIG. 3D

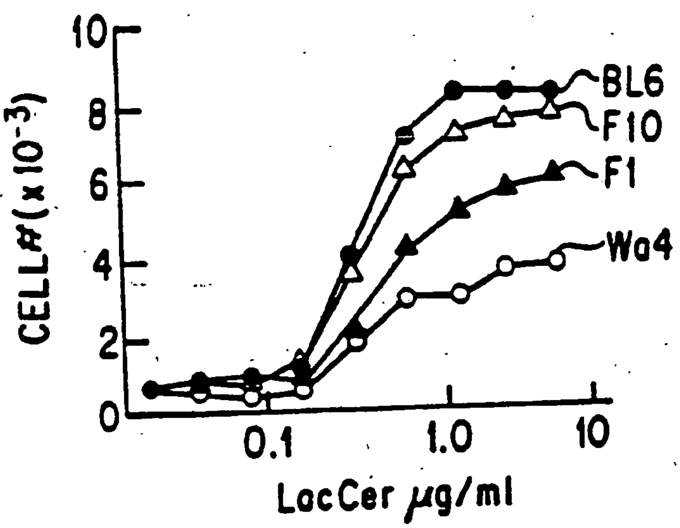


FIG. 4A

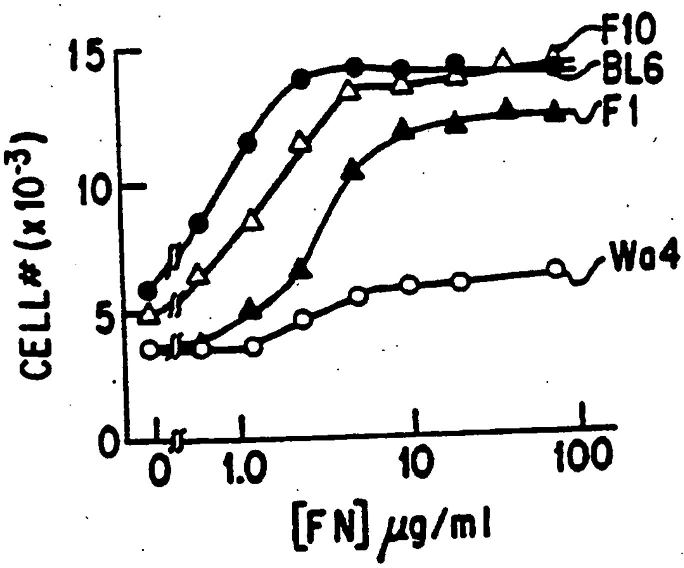


FIG. 4B

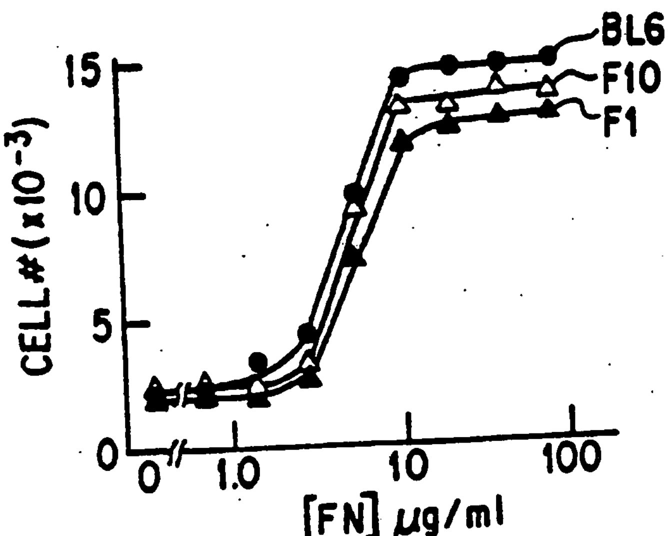


FIG. 4C

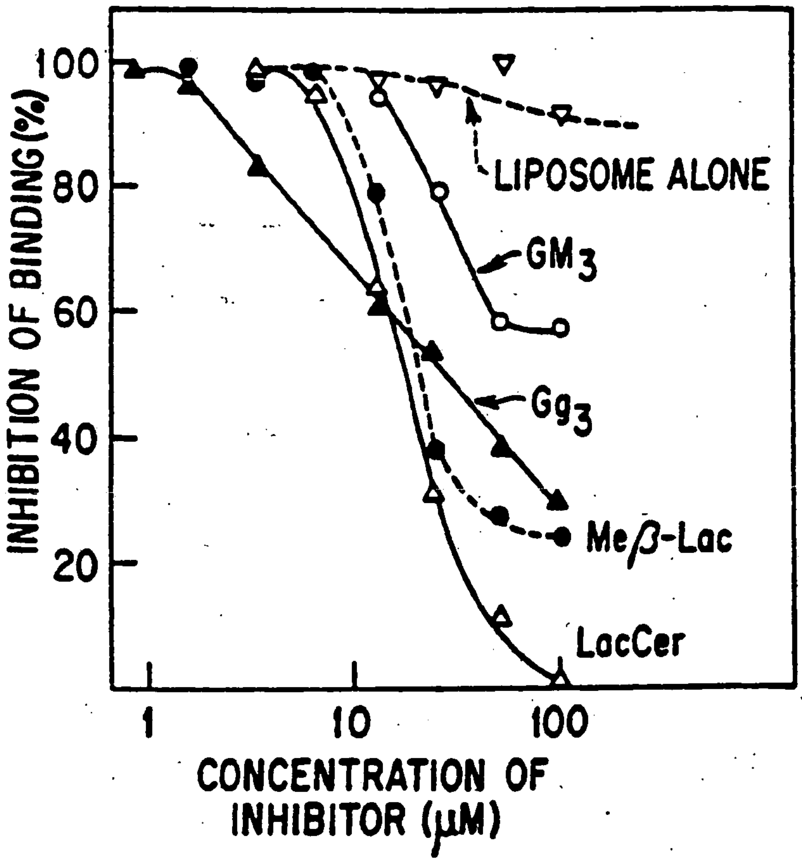

LIPOSOME ALONE
GM$_3$
Gg$_3$
Meβ-Lac
LacCer
INHIBITION OF BINDING (%)
100
80
60
40
20
1
10
100
CONCENTRATION OF INHIBITOR (μM)

FIG. 5A

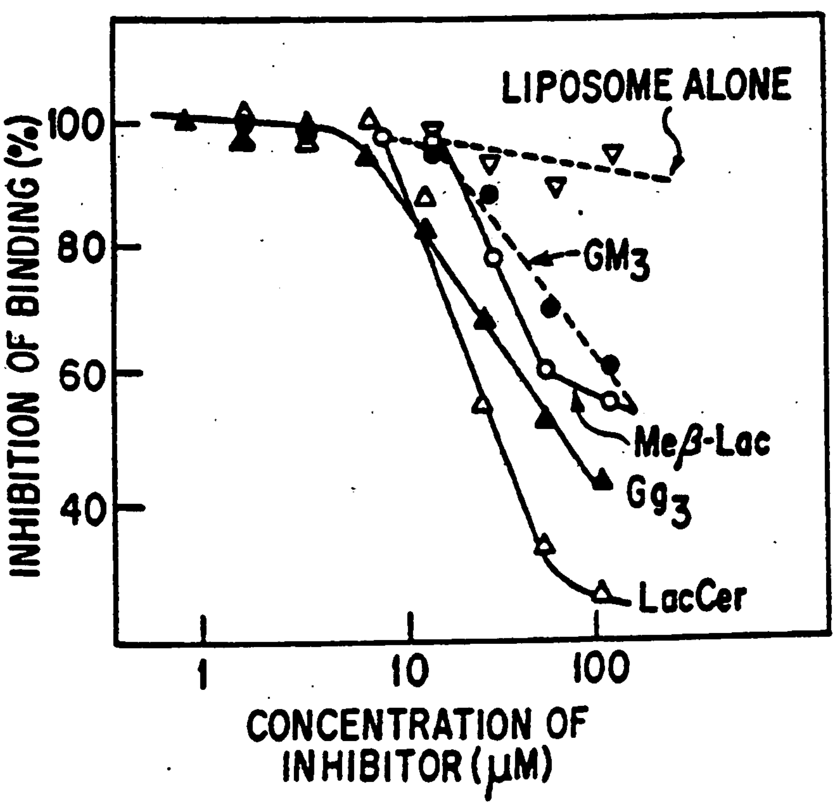

LIPOSOME ALONE
GM$_3$
Meβ-Lac
Gg$_3$
LacCer
INHIBITION OF BINDING (%)
100
80
60
40
1
10
100
CONCENTRATION OF INHIBITOR (μM)

FIG. 5B

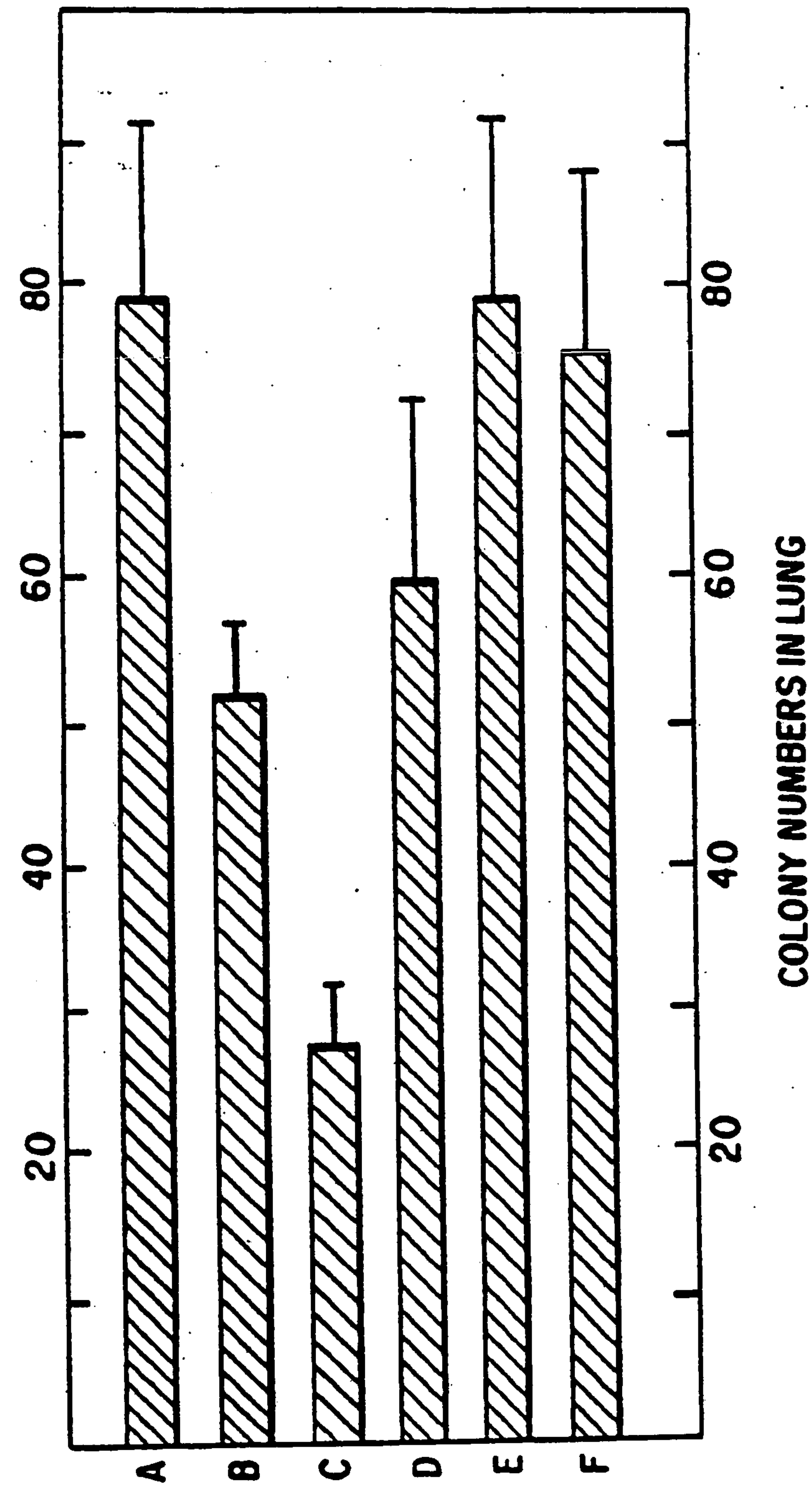


FIG. 6

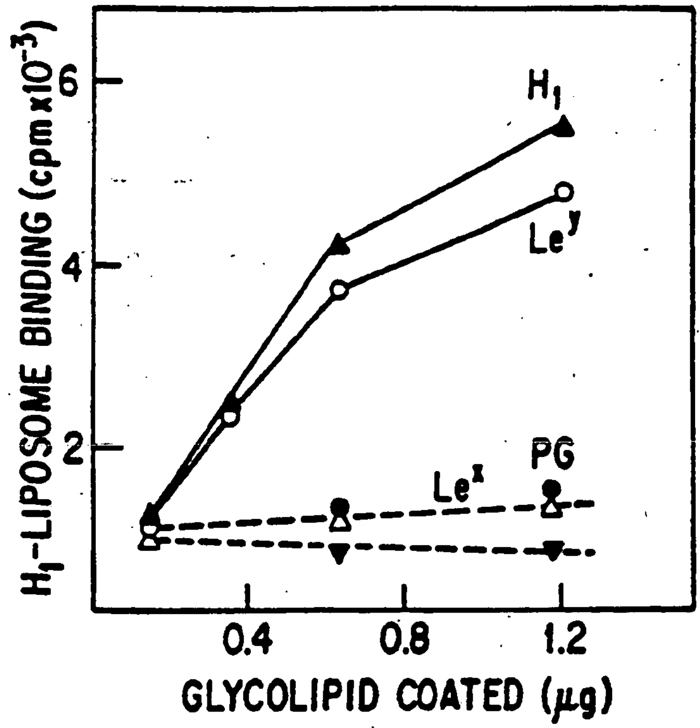

H1-LIPOSOME BINDING (cpm x10-3)
6
4
2
H1
Ley
PG
Lex
0.4
0.8
1.2
GLYCOLIPID COATED (μg)

FIG. 7A

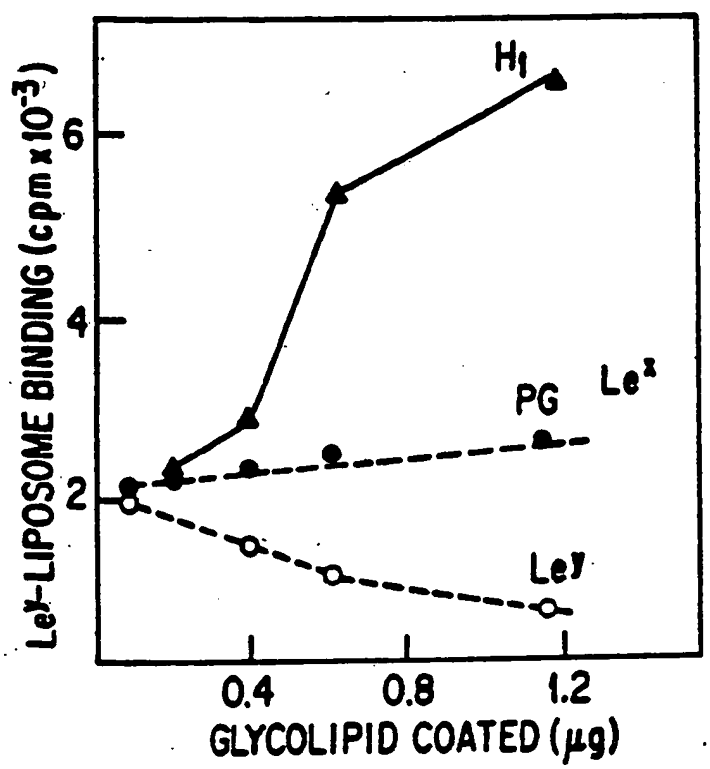

Lex-LIPOSOME BINDING (cpm x 10-3)
6
4
2
H1
PG
Lex
Ley
0.4
0.8
1.2
GLYCOLIPID COATED (μg)

FIG. 7B

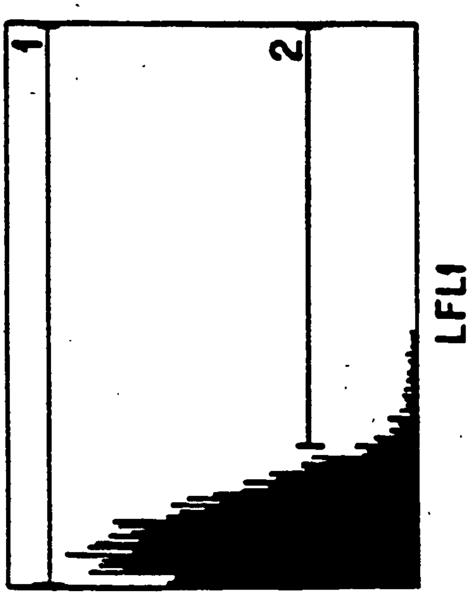


FIG. 8A

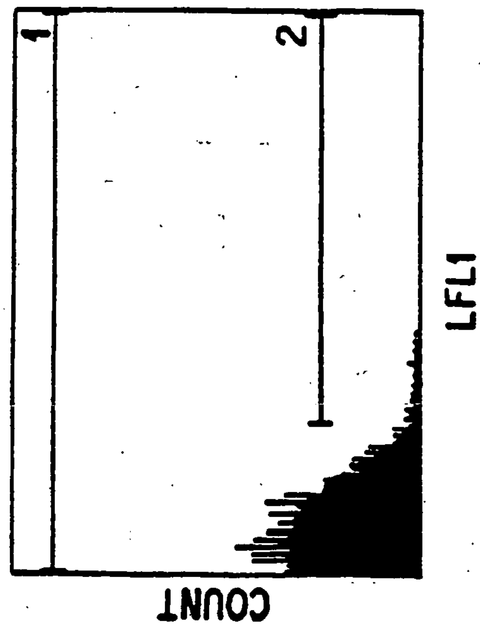


FIG. 8B

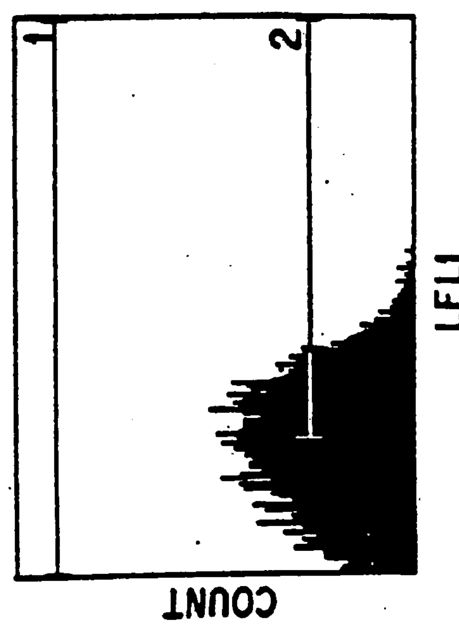


FIG. 8C

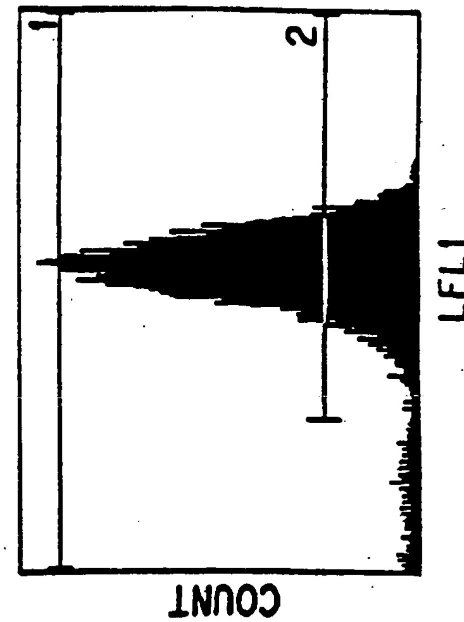


FIG. 8D

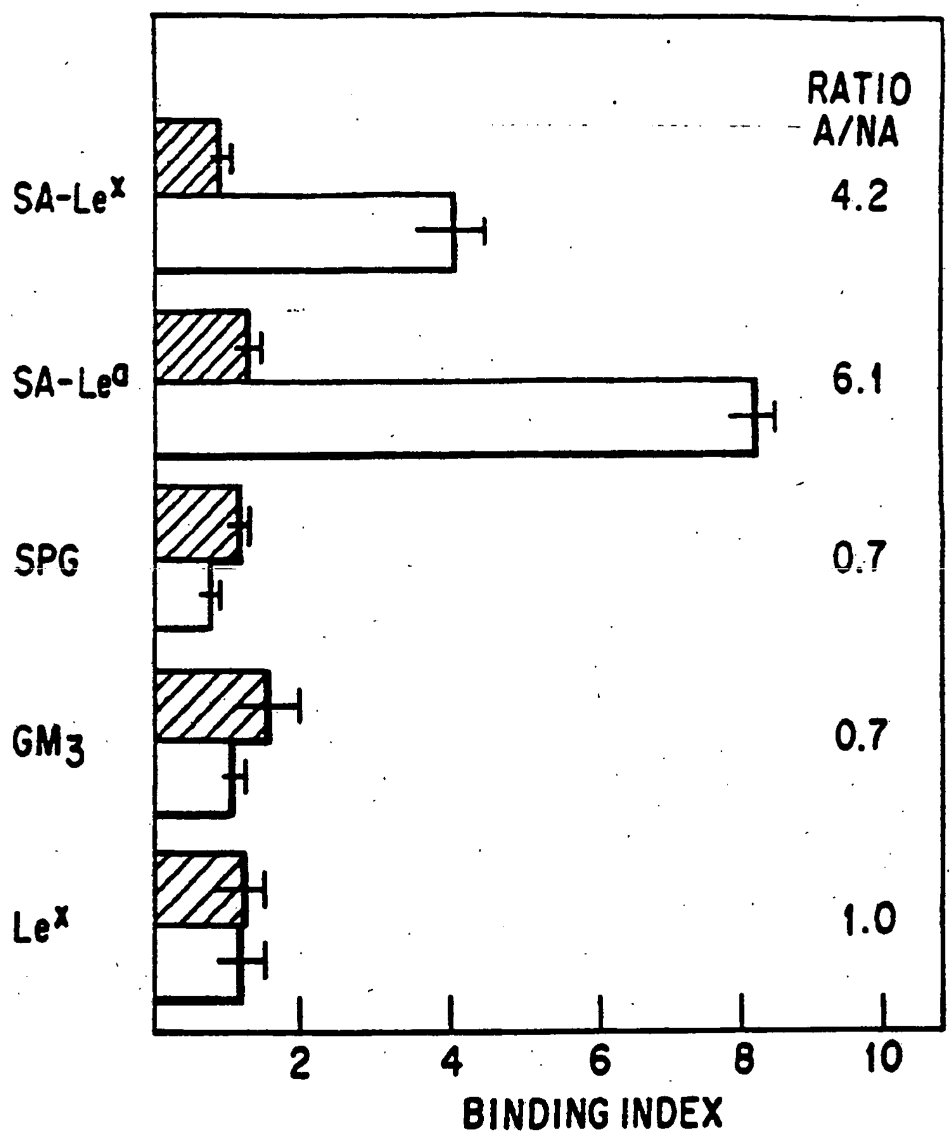

RATIO
A/NA
SA-Le^x
4.2
SA-Le^a
6.1
SPG
0.7
GM$_3$
0.7
Le^x
1.0
2
4
6
8
10
BINDING INDEX

FIG.9

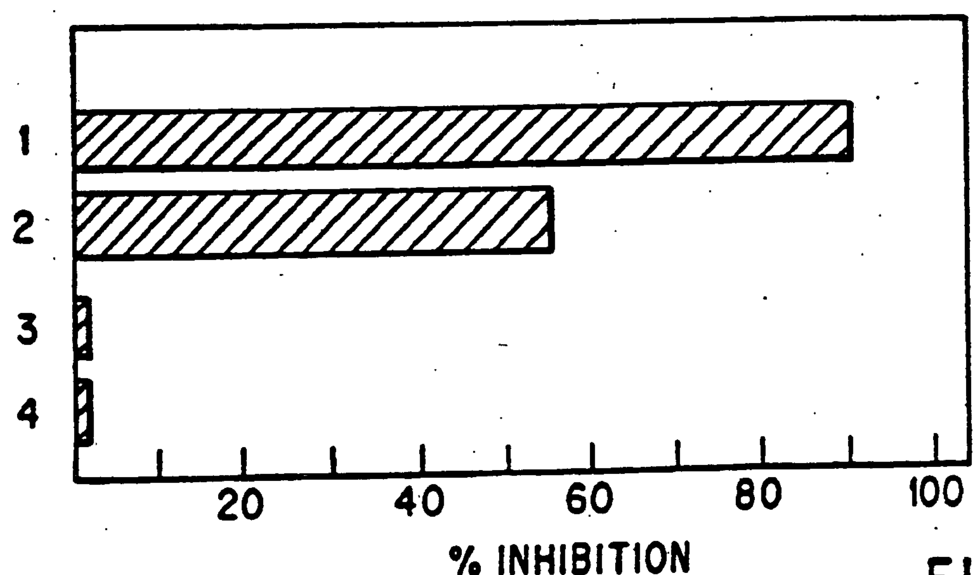

1
2
3
4
20
40
60
80
100
% INHIBITION

FIG.10

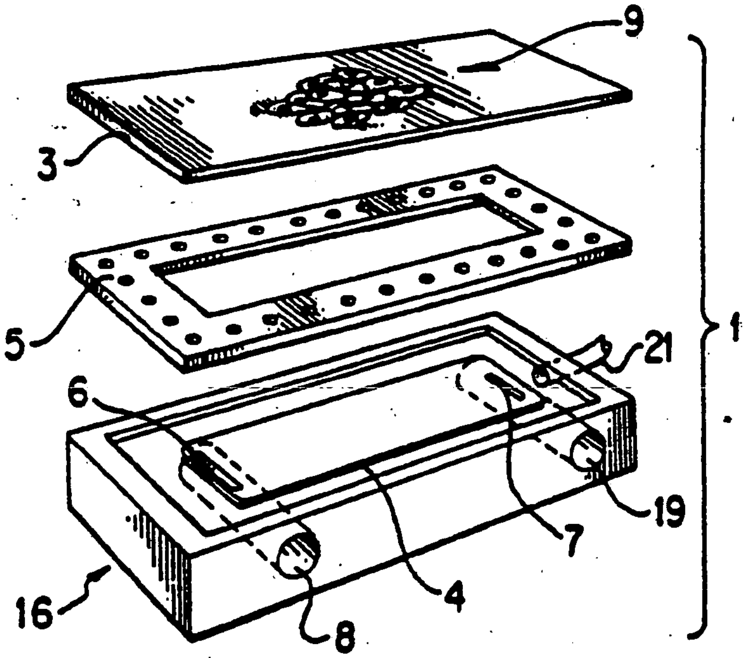


FIG. 11A

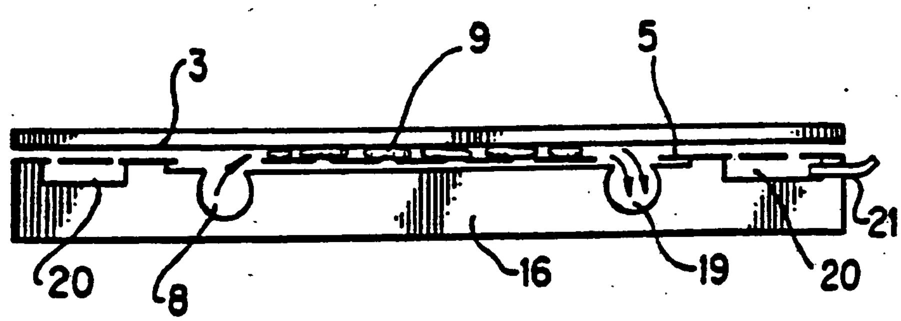


FIG. 11B

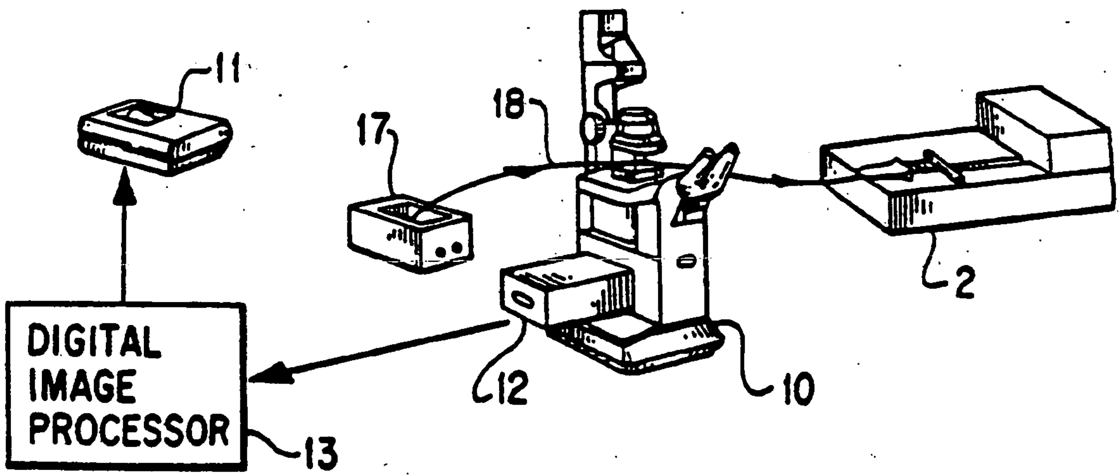


FIG. 11C

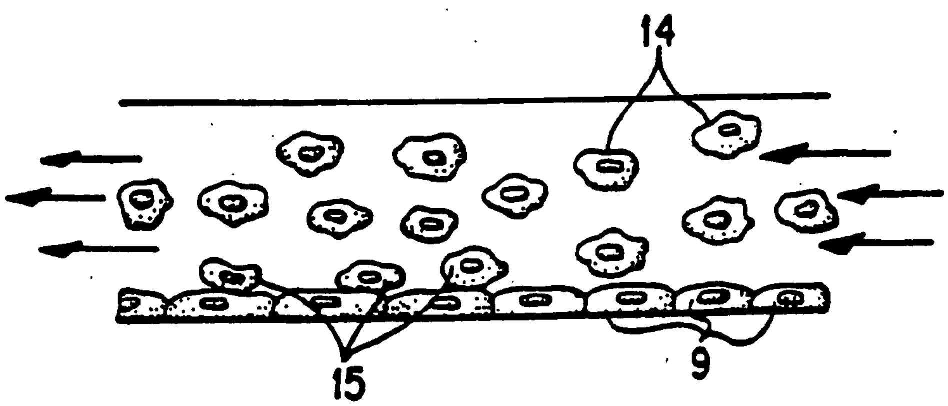
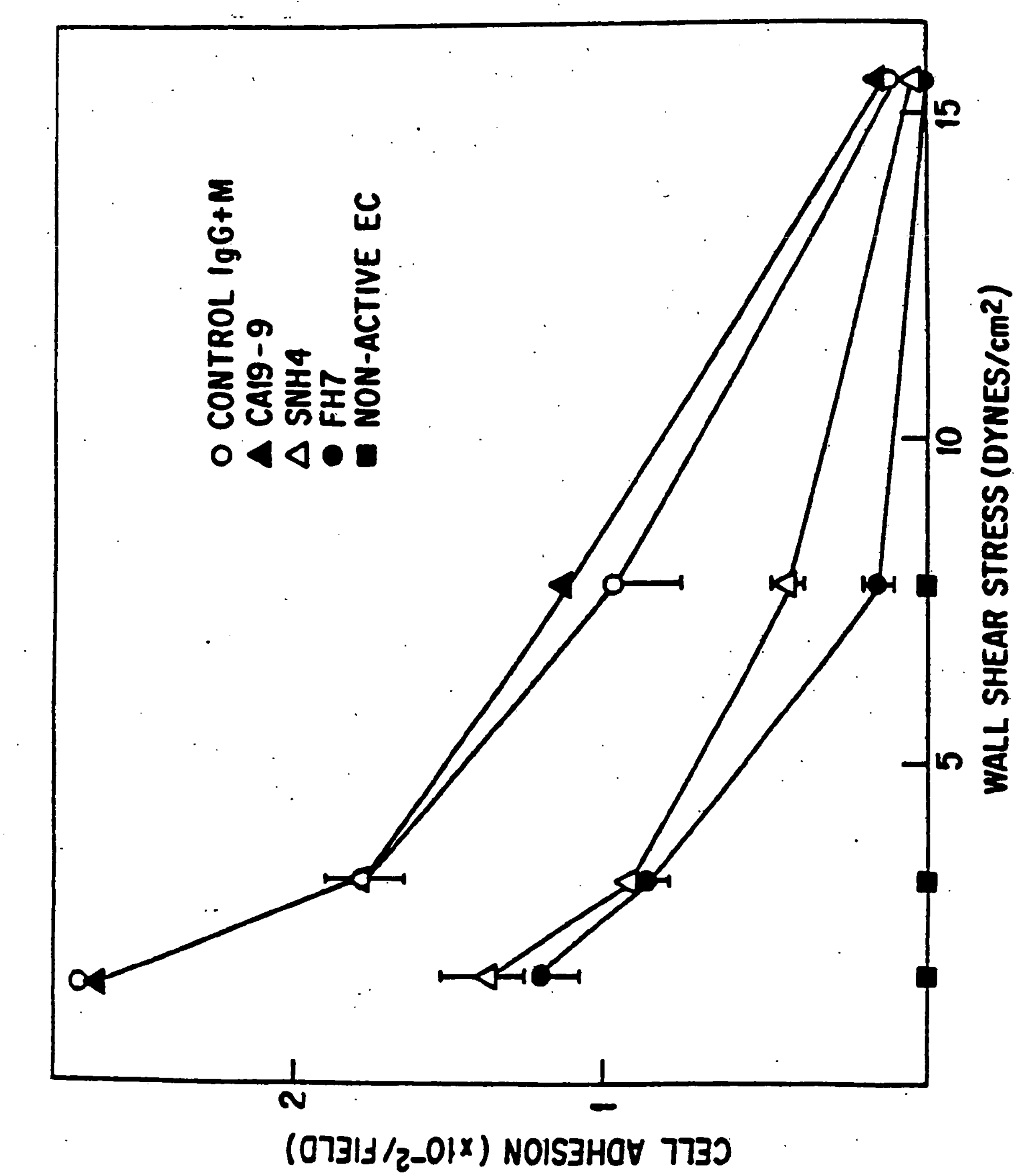


FIG. 11D

FIG.12


CONTROL IgG+M
CA19-9
SNH4
FH7
NON-ACTIVE EC
CELL ADHESION (x10⁻²/FIELD)
WALL SHEAR STRESS (DYNES/cm²)
2
1
5
10
15

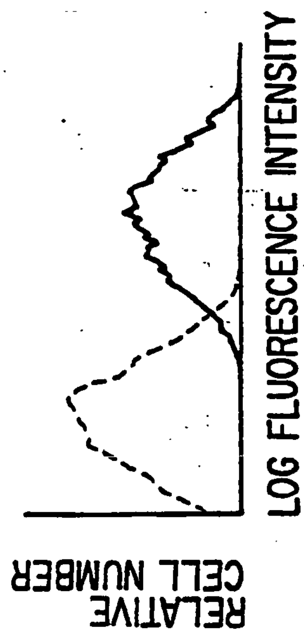


FIG. 13B

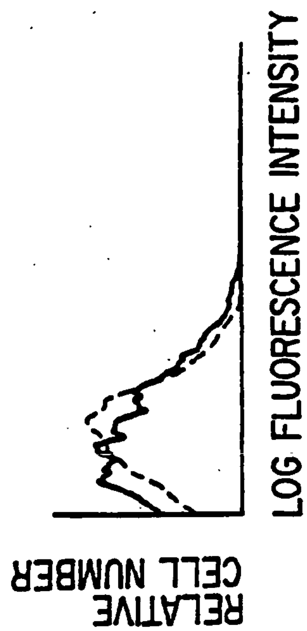


FIG. 13D

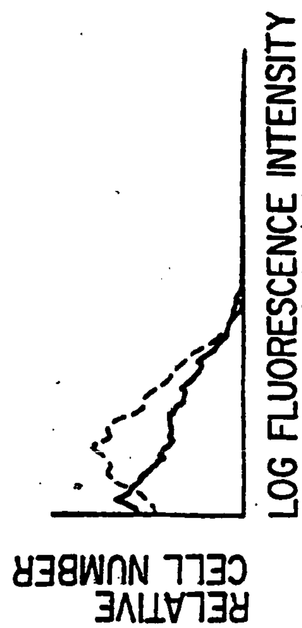


FIG. 13F

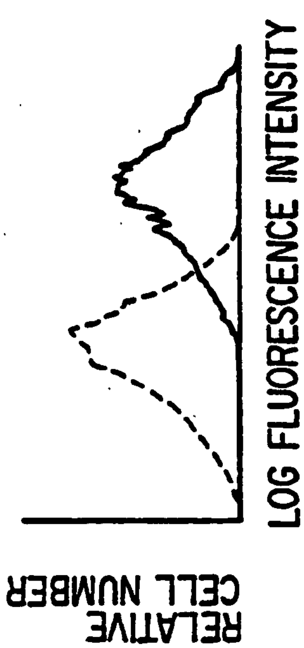


FIG. 13A

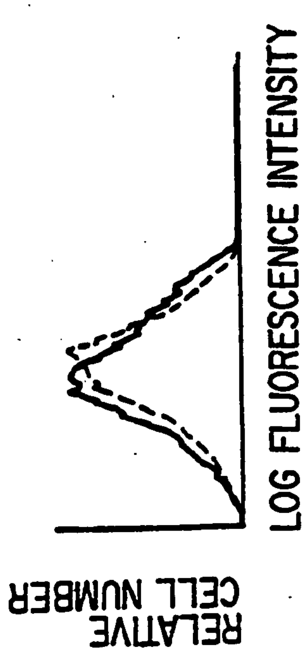


FIG. 13C

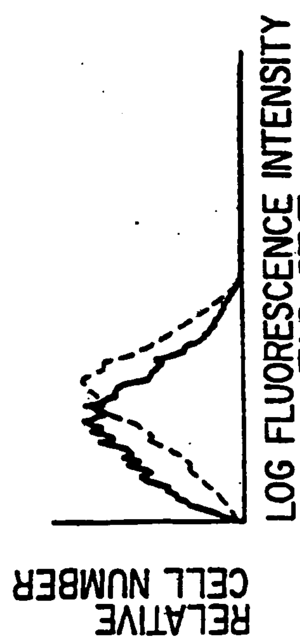


FIG. 13E

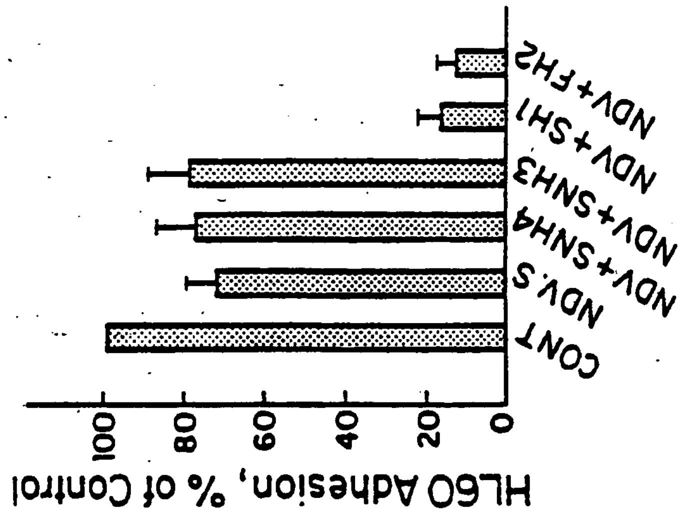


FIG. 14C

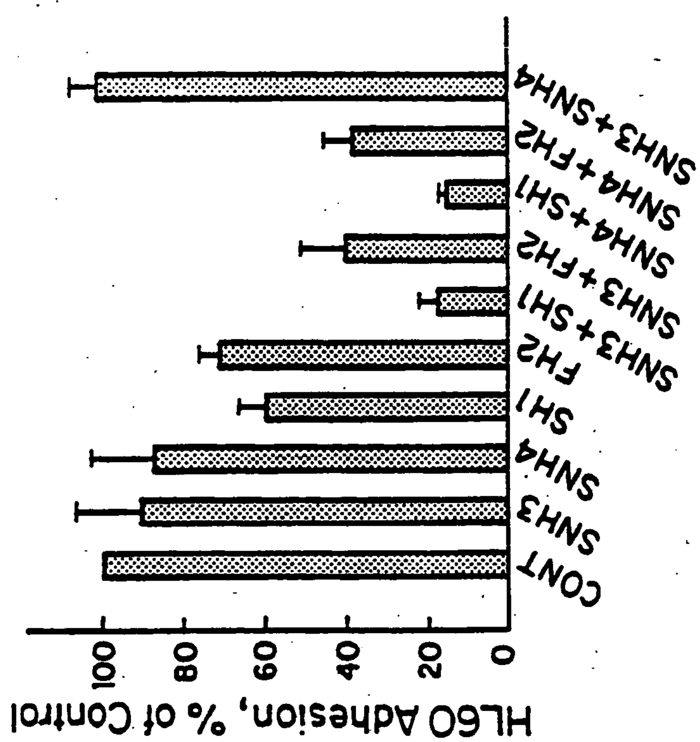


FIG. 14B

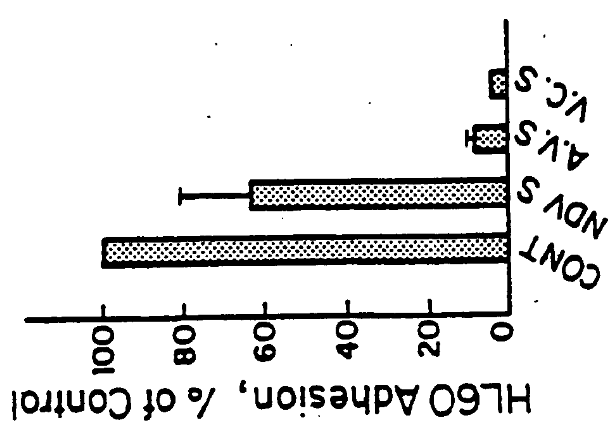


FIG.14A

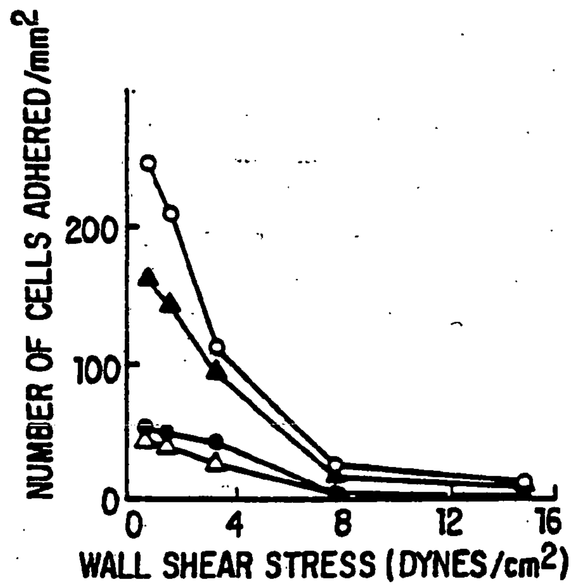


FIG.15A

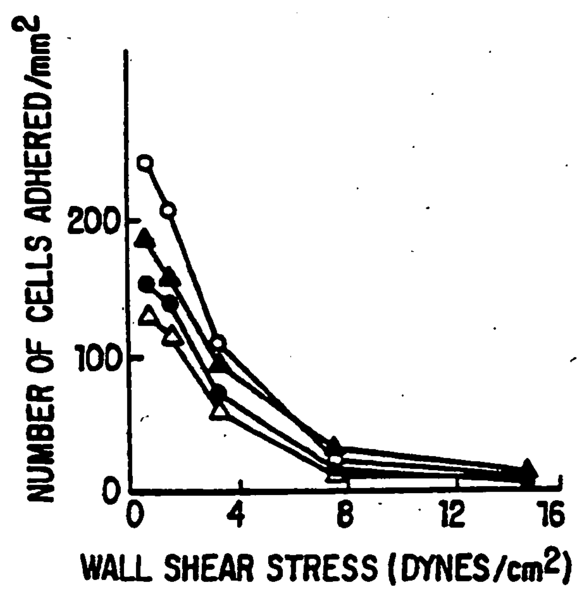


FIG. 15B

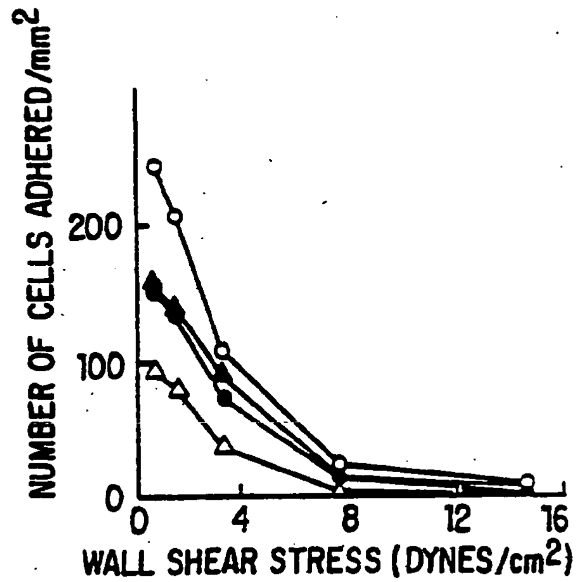


FIG. 15C

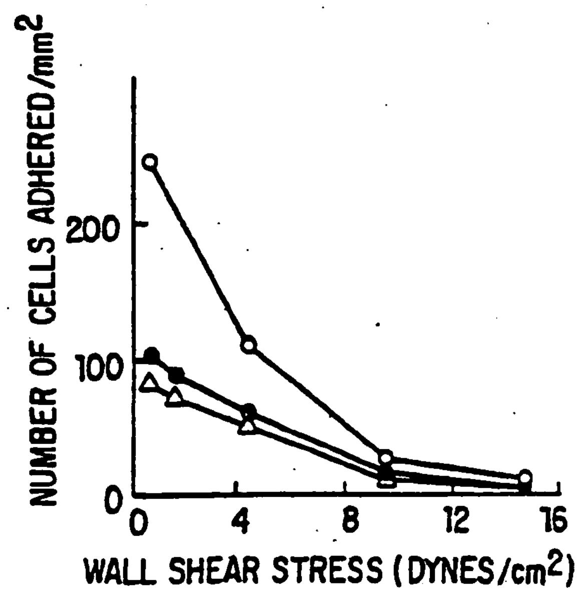


FIG. 15D

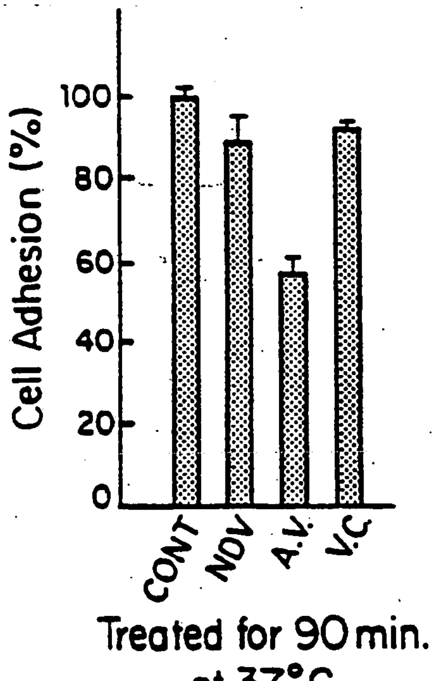


FIG. 17A

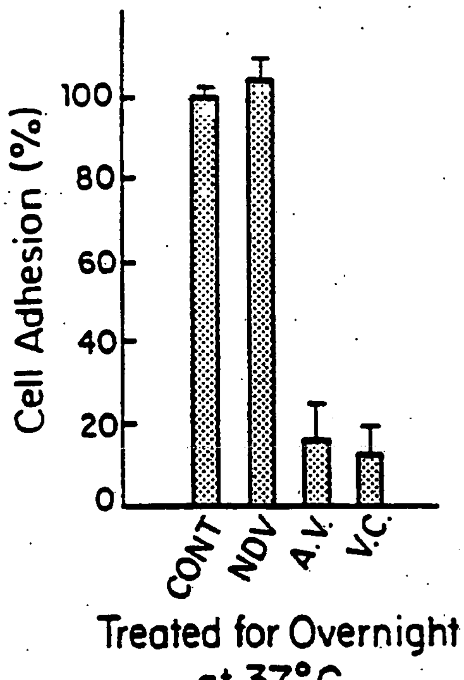


FIG. 17B

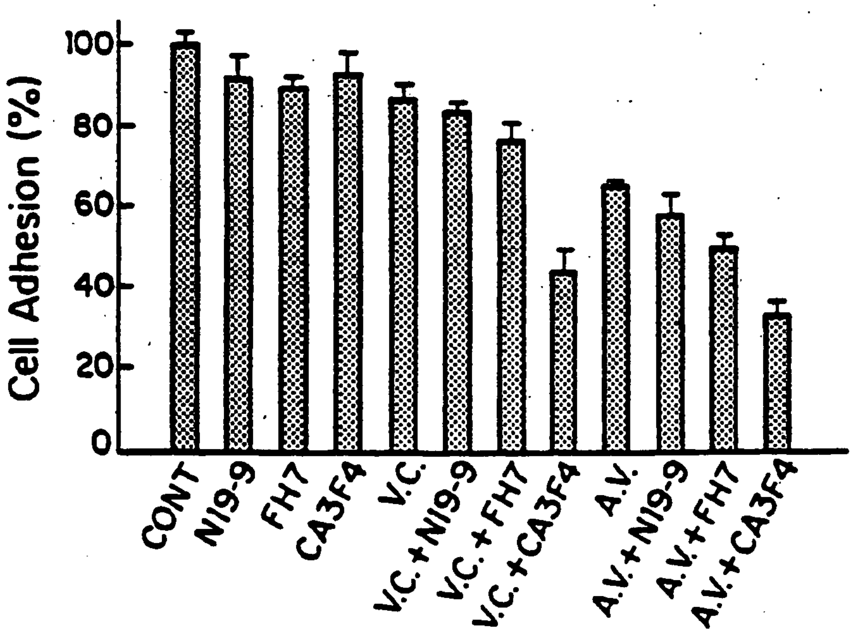


FIG. 17C

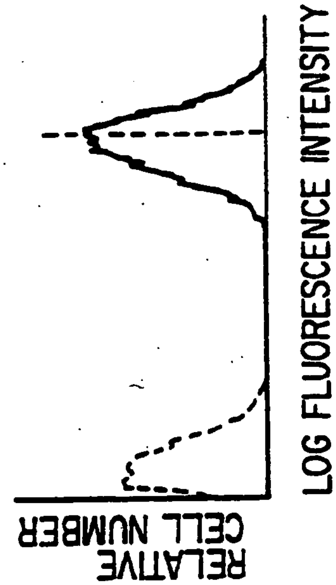


FIG. 16C

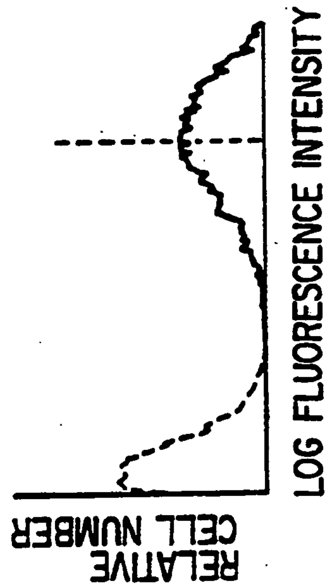


FIG. 16B

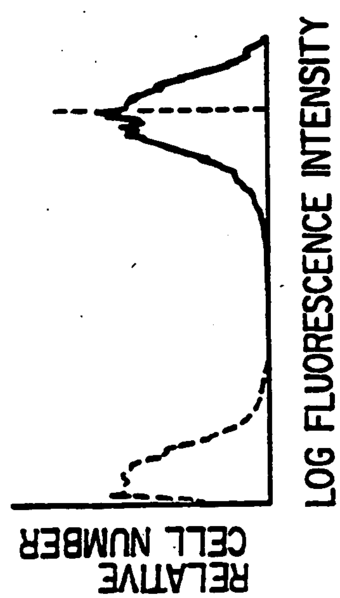


FIG. 16A

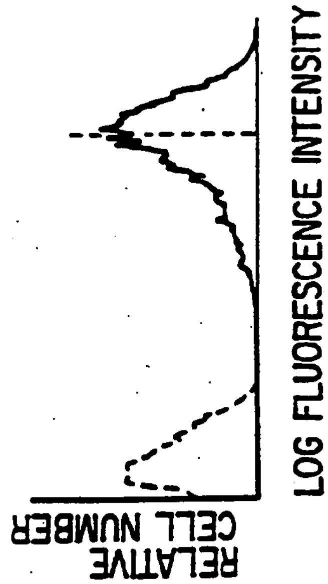


FIG. 16F

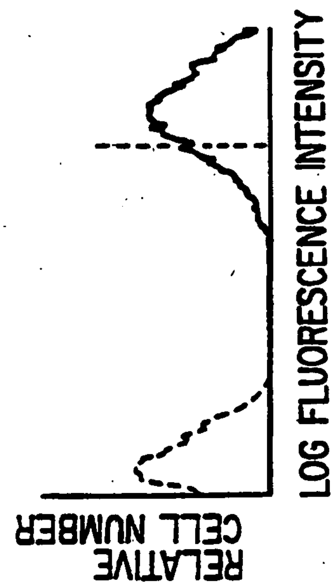


FIG. 16E

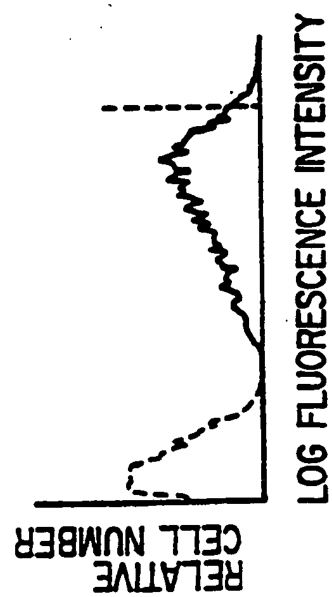


FIG. 16D

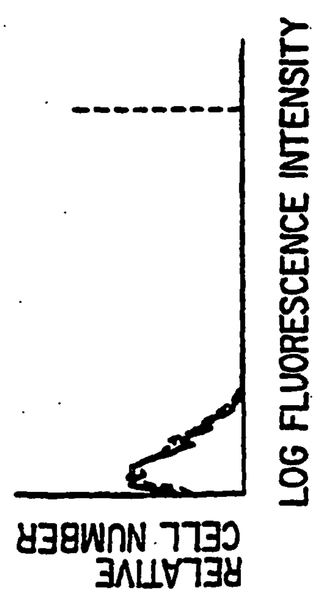


FIG. 16G

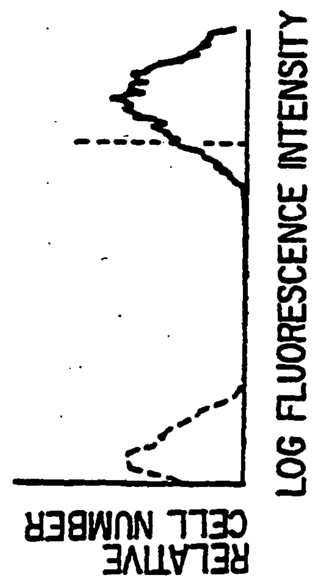


FIG. 16H

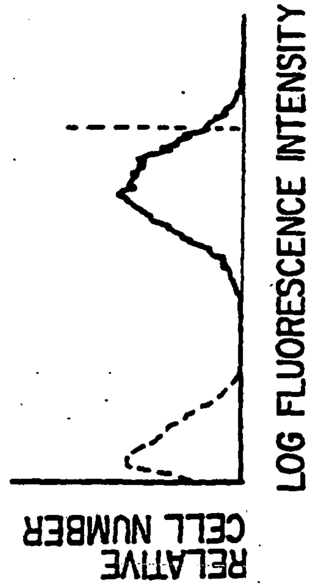


FIG. 16I

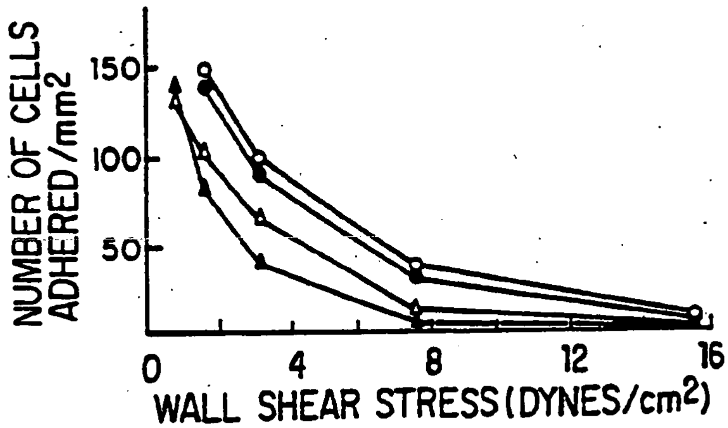


FIG. 18A

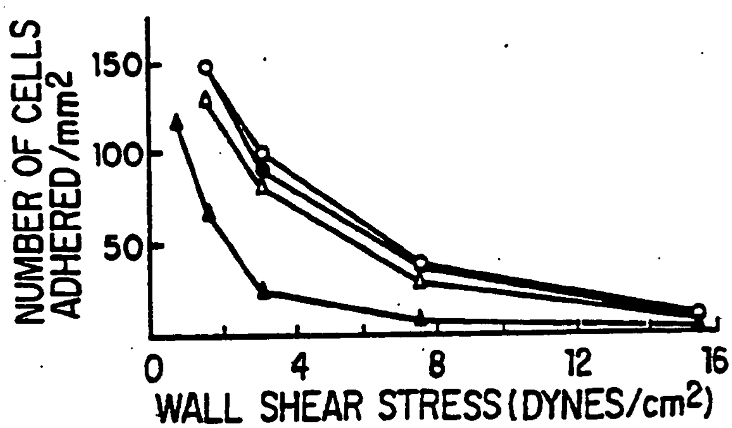


FIG. 18B

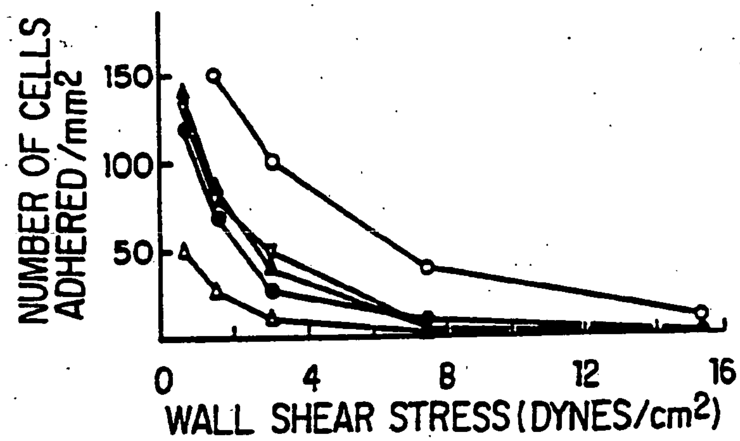


FIG. 18C

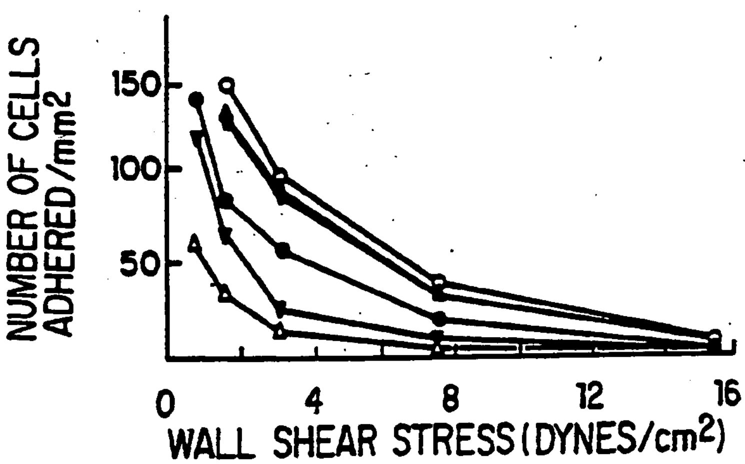


FIG. 18D

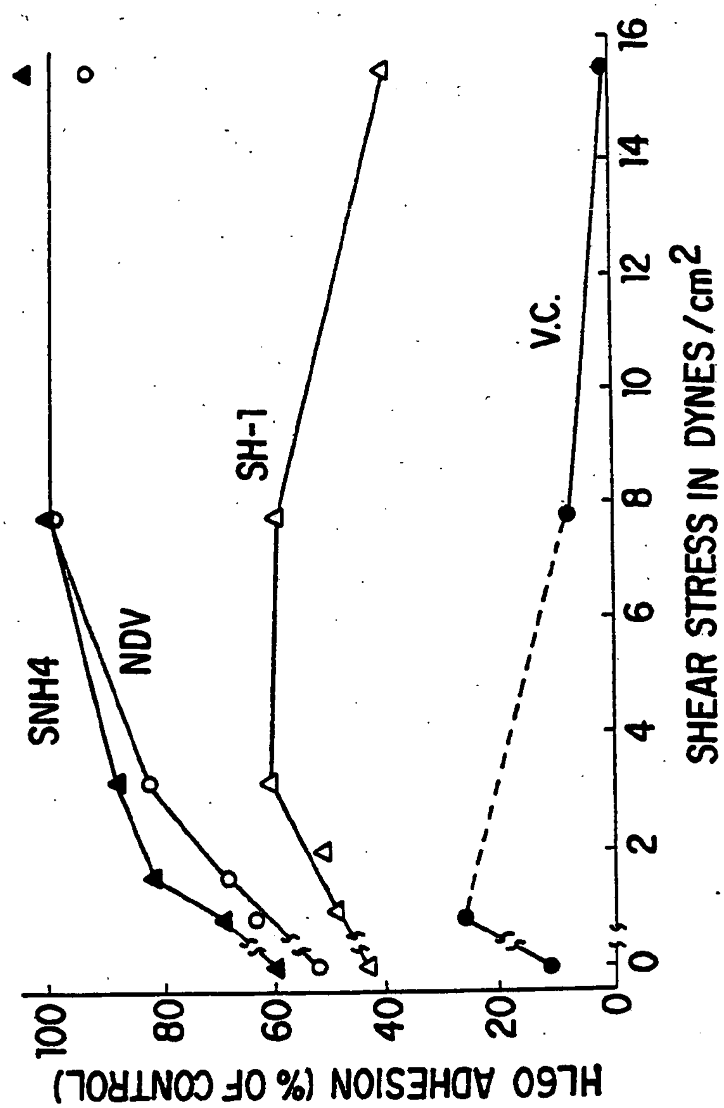

SNH4
NDV
SH-1
V.C.
HL60 ADHESION (% OF CONTROL)
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
SHEAR STRESS IN DYNES/cm^2

FIG.19

1.

```
                Fucα1
                  ↓
                  3
Galβ1→4GlcNAcβ1→6
                    Galβ1→4GlcNAcβ1→R
Galβ1→4GlcNAcβ1→3
  3               3
  ↑               ↑
SA2             Fucα1
```

2.

```
SA2             Fucα1
  ↓               ↓
  3               3
Galβ1→4GlcNAcβ1→6
                    Galβ1→4GlcNAcβ1→R
Galβ1→4GlcNAcβ1→3
  3               3
  ↑               ↑
SA2             Fucα1
```

3.

```
        Fucα1
          ↓
          3
Galβ1→4GlcNAcβ1→6
                   GalNAcα1→O-Ser/Thr
     SA2→xGalβ1→3
```

FIG. 20

```
4.            Fucα1
                ↓
                4
   Galβ1→3GlcNAcβ1→6
                        Galβ1→4GlcNAcβ1→R
   Galβ1→3GlcNAcβ1→3
     3          4
     ↑          ↑
    SA2       Fucα1
```

```
5.       Galβ1→4GlcNAcβ1→6
                            Galβ1→4GlcNAcβ1→3Galβ1→R
         Galβ1→4GlcNAcβ1→3
           3
           ↑
          SA2
```

```
6.        SA2
           ↓
           3
         Galβ1→4GlcNAcβ1→6
                            Galβ1→4GlcNAcβ1→3Galβ1→R
         Galβ 1→4GlcNAcβ1→3
           3
           ↑
          SA2
```

FIG. 20 CONT.

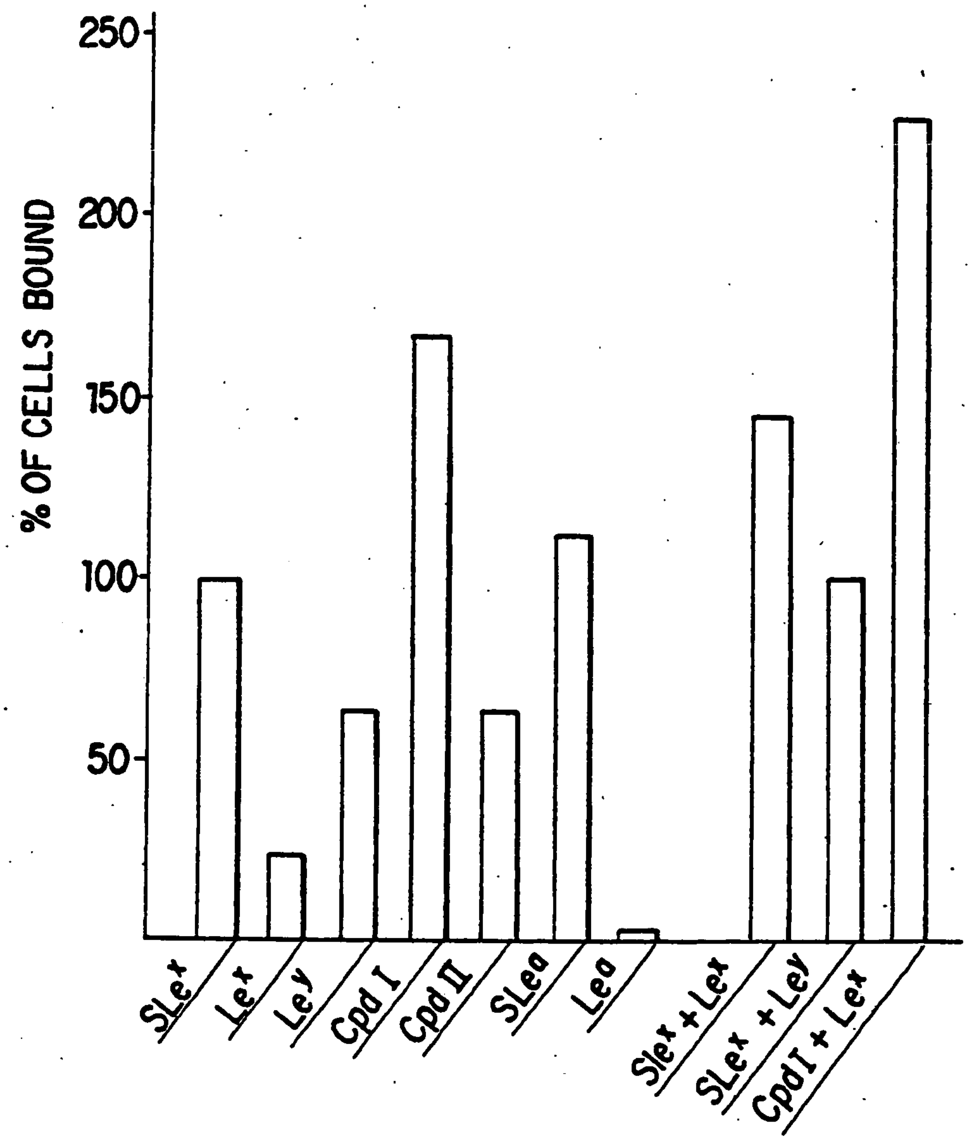

250
200
150
100
50
% OF CELLS BOUND
SLex
Lex
Ley
Cpd I
Cpd II
SLea
Lea
Slex + Lex
SLex + Ley
CpdI + Lex

FIG. 21A

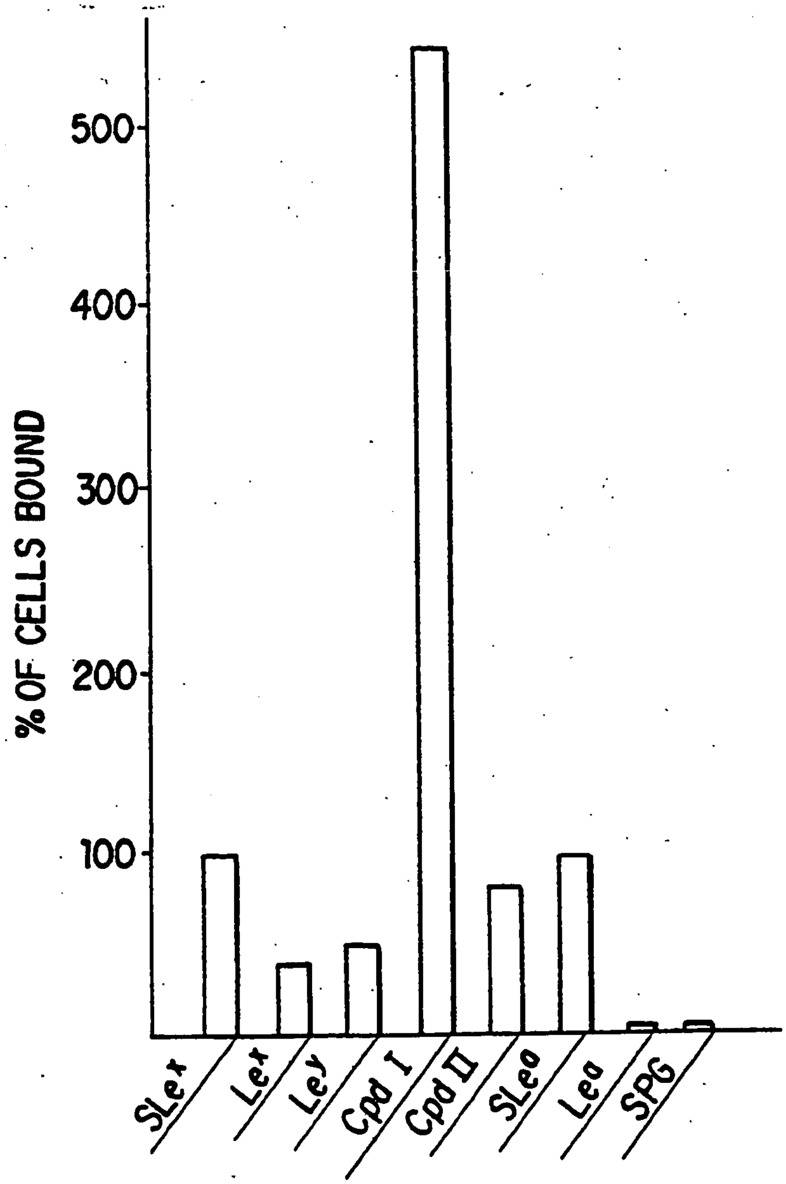

% OF CELLS BOUND
500
400
300
200
100
SLex
Lex
Ley
Cpd I
Cpd II
SLea
Lea
SPG

FIG. 21B

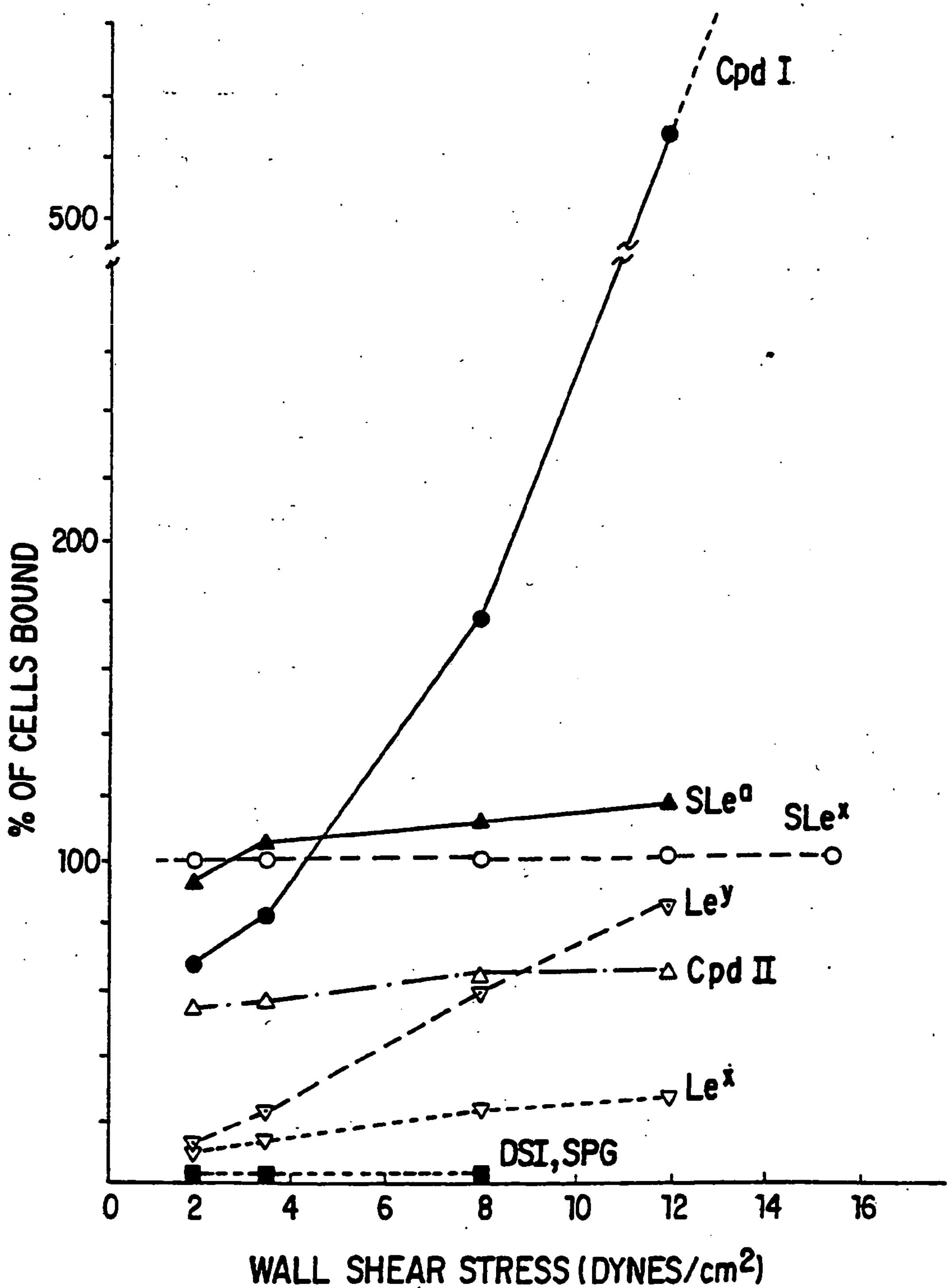

Cpd I
500
200
100
% OF CELLS BOUND
SLe^a
SLe^x
Le^y
Cpd II
Le^x
DSI, SPG
0
2
4
6
8
10
12
14
16
WALL SHEAR STRESS (DYNES/cm^2)

FIG. 22

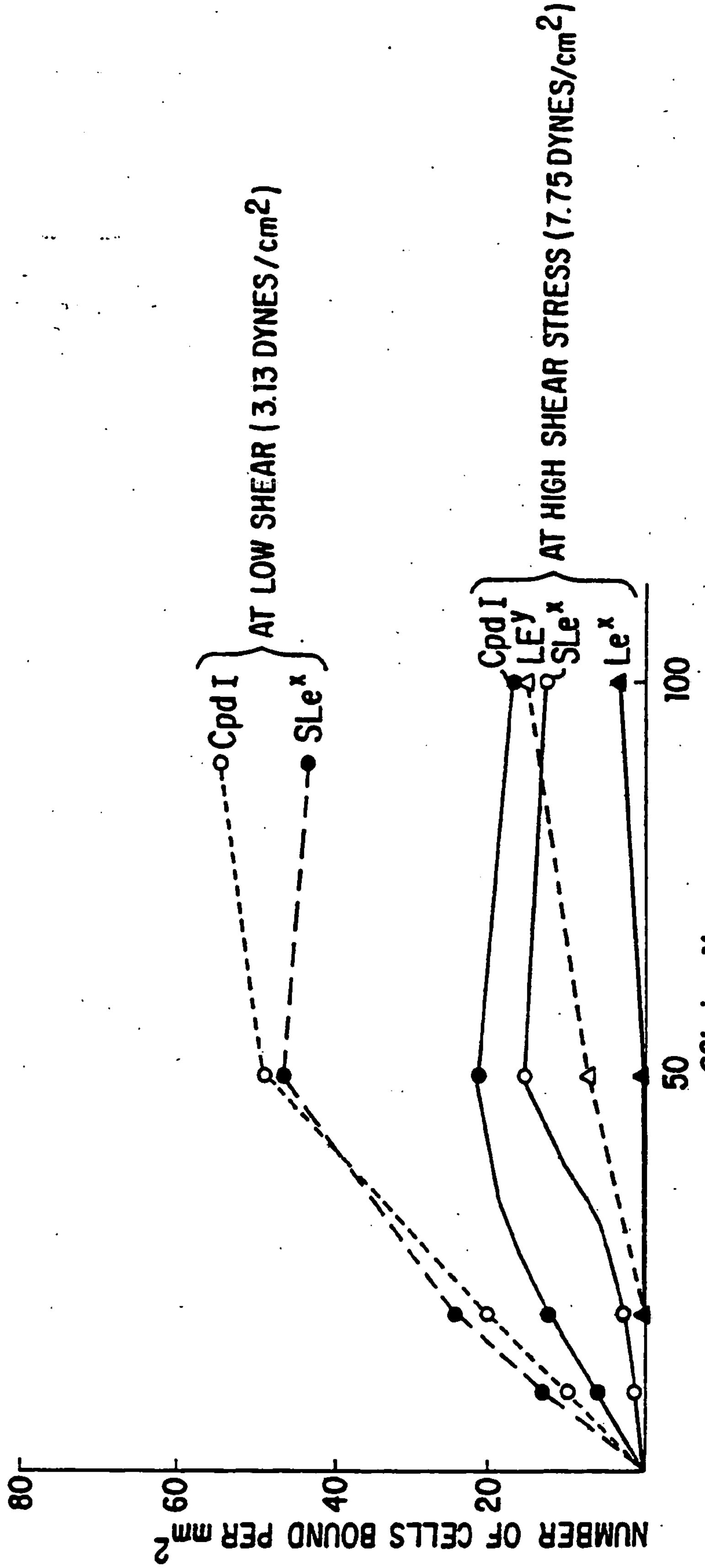

AT LOW SHEAR (3.13 DYNES/cm2)
Cpd I
SLex
AT HIGH SHEAR STRESS (7.75 DYNES/cm2)
Cpd I
LEy
SLex
Lex
NUMBER OF CELLS BOUND PER mm2
80
60
40
20
50
100
GSL in μM

FIG. 23

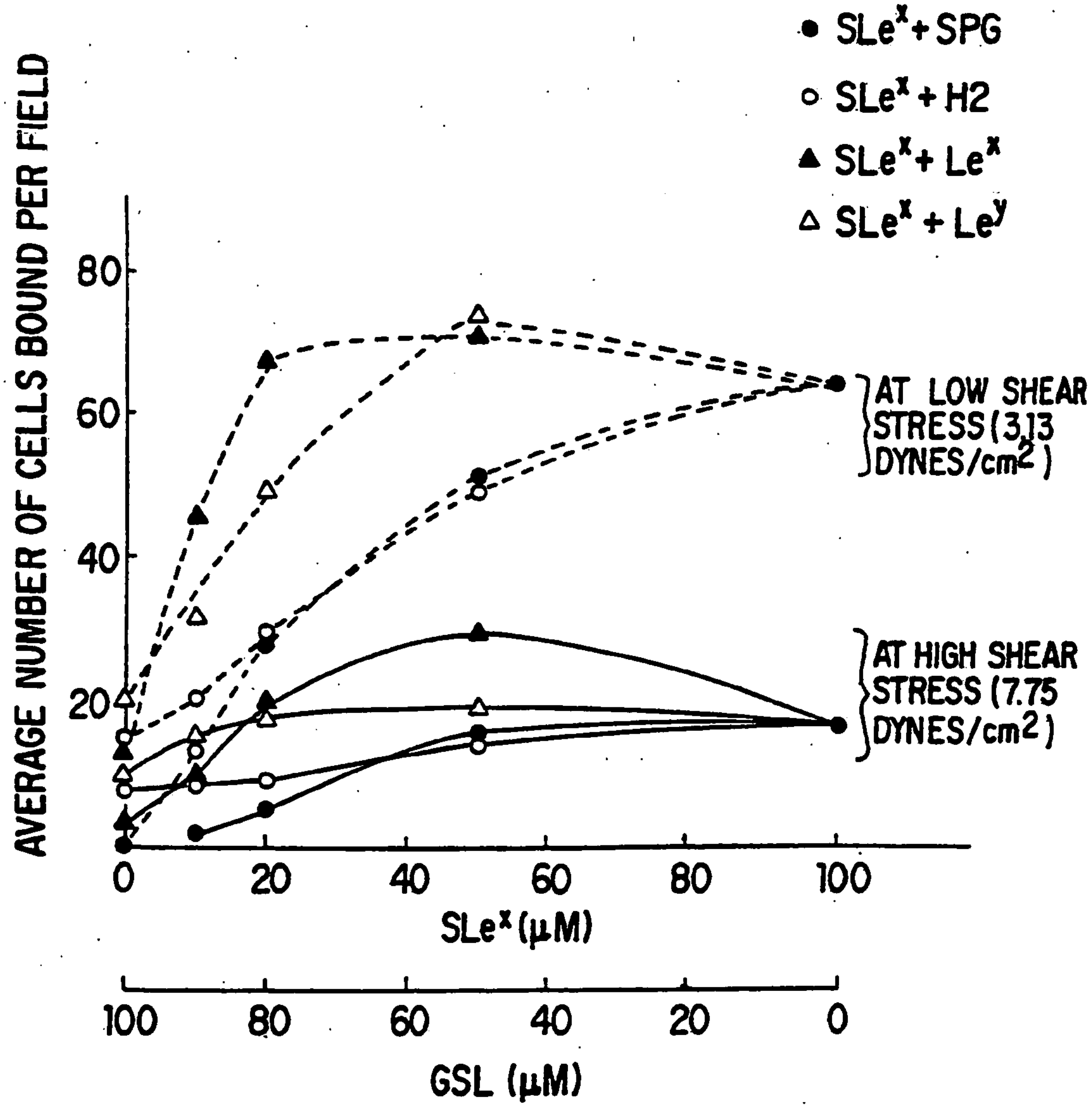


FIG. 24

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/01375

A. CLASSIFICATION OF SUBJECT MATTER
IPC(5) :C07H 3/06
US CL :536/1.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 536/1.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Dialog, APS: Search Terms: Inventor Names
Structure search of STN made by library

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Cancer Research, Volume 48, issued 15 January 1988, T.S. Kim et al., "Le(x) and Le(y) antigen expression in human pancreatic cancer", pages 475-483, entire document.	3-7
Y	J. Biol. Chem., Volume 264, No. 31, issued 05 November 1989, E.D. Nudelman et al., "A series of disialogangliosides with binary 2-3 sialosyllactosamine structure, defined by monoclonal antibody NUH2, are oncodevelopmentally regulated antigens", pages 18719-18725, entire document.	3-7

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be part of particular relevance
"E" earlier document published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search
13 JULY 1993

Date of mailing of the international search report
15 JUL 1993

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. NOT APPLICABLE

Authorized officer
F. CHRISTOPHER EISENSCHENK
Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation f document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Clin. Exp. Metastasis, Volume 9, No. 3, issued May-June 1991, H. Inufusa et al., "Human lung adenocarcinoma cell lines with different lung colonization potential and a correlation between expression of sialosyl dimeric Le(x) and LCP", abstract, entire document.	3-7
Y	J. Biol. Chem., Volume 266, No. 13, issued 05 May 1991, M.R. Stroud et al., "Extended type 1 chain glycosphingolipids: dimeric Le(a) as human tumor associated antigen", pages 8439-8446.	3-7

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
(Telephone Practice)
Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
3-7

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

I. Claim 1, drawn to a carbohydrate having the formula of claim 1, classified in Class 536, subclass 1.1.

II. Claim 2, drawn to a carbohydrate of the formula of claim 2, classified in Class 536, subclass 1.1.

III. Claims 3-7, drawn to a carbohydrate of the formula of claim 3, classified in Class 536, subclass 1.1.

IV. Claim 8, drawn to a carbohydrate of the formula of claim 8, classified in Class 536, subclass 1.1.

V. Claims 9-12 and 24-25, drawn to antibodies specific for the carbohydrates of claims 1-8 and methods of therapy using these anubodies, classified in Class 530, subclass 388.8 and Class 424, subclass 85.8.

VI. Claims 13-18, drawn to compositions comprising two carbohydrates and liposomes, classified in Class 536, sublass 1.1.

VII. Claims 19-23, drawn to a composition comprising at least two antibodies specific for the carbohydrate compositions of Group VI, classified in Class 530, subclass 388.8.

VIII. Claims 26-35, drawn to a method of interrupting cellular interactions using antibodies which bind carbohydrates associated with the ELAM-1 molecule, classified in Class 424, subclass 85.8.

IX. Claims 36-42, drawn to a method of interrupting cellular interactions using antibody which specifically binds ELAM-1, classified in Class 424, subclass 85.8.